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RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE

A study of renal disease in Wegener`s granulomatosis and in primary Sjögren`s syndrome

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Trondheim, January 2001

Knut Aasarød

List of papers

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals.

I. **Aasarød K, Iversen BM, Hammerstrøm J, Bostad L, Vatten L, Jørstad S. Wegener`s granulomatosis : clinical course in 108 patients with renal involvement** *Nephrol Dial Transplant* **2000;15:611-618**

II **Aasarød K, Iversen BM, Hammerstrøm J, Bostad L, Jørstad S. Clinical outcome in patients with Wegener`s granulomatosis treated with plasma exchange. (Accepted for publication)**

III **Aasarød K, Bostad L, Hammerstrøm J, Jørstad S, Iversen BM. Renal histopathology and clinical course in 94 patients with Wegener`s granulomatosis** *Nephrol Dial Transplant* **2001;16:953-960**

IV **Aasarød K, Bostad L, Hammerstrøm J, Jørstad S, Iversen BM. Wegener`s granulomatosis: Inflammatory cells and markers of repair and fibrosis in renal biopsies-a clinicopathological study. (Accepted for publication)**

V **Aasarød K, Haga H-J, Berg KJ, Hammerstrøm J, Jørstad S. Renal involvement in primary Sjögren`s syndrome.** *Q J Med* **2000; 93:297-304**

Abbreviations

α-SMA	α-Smooth Muscle Actin
ACR	American College of Rheumatology
ALP	Alkaline Phosphatase
ANA	Anti-Nuclear Antibodies
ANCA	Anti Neutrophil Cytoplasmic Autoantibodies
ATD	Acute Tubular Damage
BC	Bowman`s Capsule
CS	Corticosteroids
CYC	Cyclophosphamide
DDAVP	1-Desamino-8-D-Arginine-Vasopressin
dRTA	Distal Renal Tubular Acidosis
ELISA	Enzyme-Linked Immunosorbent Assay
ESRD	End Stage Renal Disease
GFR	Glomerular Filtration Rate
IIF	Indirect Immunofluorescence
MP	Methylprednisolon
MPA	Microscopic Polyangiitis
MPO	Myeloperoxidase
MRR	Mortality Risk Ratio
NAG	N-Acetyl-β-Glucosaminidase
NPV	Negative Predictive Value
PAS	Periodic Acid-Schiff
PCP	Pneumocystis Carinii Pneumonia
PE	Plasma Exchange
PPV	Positive Predictive Value
PR3	Proteinase 3
PSS	Primary Sjögren`s Syndrome
RPGN	Rapidly Progressive Glomerulonephritis
WG	Wegener`s Granulomatosis

General introduction

Renal involvement is found in several inflammatory rheumatic diseases and when it occurs it very often has a negative effect on morbidity and long-term patient survival. This is particularly true in systemic vasculitic syndromes (1-3) and in systemic lupus erythematosus (4, 5), where renal disease is the most important limiting factor in disease recovery. Renal involvement in systemic sclerosis, mixted connective tissue disease, Henoch Schönlein purpura, and renal amyloidosis secondary to ankylosing spondylitis, rheumatoid arthritis or psoriasis arthritis, also heralds a more severe outcome for the patients (6-10). In these diseases renal involvement very often becomes overt with proteinuria, hematuria, erythrocyte casts and frequently with significantly elevated serum creatinine concentration. Many patients will eventually develop end stage renal disease requiring dialysis or renal transplantation.

On the other end of the spectrum is primary Sjögren`s syndrome where renal involvement is mostly silent, very rarely leads to chronic renal impairment and in general does not seem to have a detrimental effect on patient survival (11). Renal disease in primary Sjögren`s syndrome is not without clinical implications however, as it is associated with renal tubular acidosis (12), renal calculus formation (13), and rarely with life threatening hypokalemia (14, 15).

The aim of the present investigation has been to elucidate different aspects of renal involvement in Wegener`s granulomatosis and in primary Sjögren`s syndrome. In the study of Wegener`s granulomatosis emphasis has been put on the clinical course during follow-up, with special reference to patient morbidity and mortality, and also on renal histopathology and a search for a possible prognostic value of the kidney biopsy. For primary Sjögren`s syndrome focus is on renal glomerular and tubular pathophysiology.

Wegener`s granulomatosis (WG)

The first reports of WG can be credited Heinz Klinger and Frederik Wegener (16) in the 1930`s. Klinger described a 70-year old physician suffering from fever and maxillary sinusitis, who subsequently developed suppurative nasal discharge and deformity, arhritis, pulmonary vasculitis and nephritis (17). Later studies have shown that the disease is chronic and frequently relapsing with a substantial morbidity and mortality. In the 1950`s the median patient survival was 5 months and one year mortality was 80% (18). Basically WG is characterised by sterile inflammatory injury to the upper and lower airways and, in most instances, by glomerulonephritis (19-21). The inflammatory changes typically include necrosis, granuloma formation and vasculitis in small to medium sized vessels (22).

Classification and epidemiology

WG is a multisystem disease of unknown etiology belonging to the group of systemic vasculitides, now often referred to as ANCA-associated vasculitis (23). The classification and the nomenclature of the vasculitides are controversial. The most commonly used classification criteria are the ones established by the American College of Rheumatology (ACR) (20) mostly based on clinical symptoms and findings (Table 1).

Table 1. The American College of Rheumatology 1990 criteria for classification of Wegener`s granulomatosis*

Criterion	Definition
1. Nasal or oral inflammation	Development of painful or painless oral ulcers or purulent or bloody nasal discharge
2. Abnormal chest radiograph	Chest radiograph showing the presence of nodules, fixed infiltrates, or cavities
3. Urinary sediment	Microhematuria (>5 red blood cells per high power field) or red cell casts in urine sediments
4. Granulomatous inflammation on biopsy	Histologic changes showing granulomatous inflammation within the wall of an artery or in the perivascular or extravascular area (artery or arteriole)

*For purposes of classification, a patient shall be said to have Wegener`s granulomatosis if at least 2 of these 4 criteria are present. The presence of any 2 or more criteria yields a sensitivity of 88.2% and a specificity of 92.0% (Reference 20)

Classification criteria select those clinical findings that both identify the disease and separate it from others. As a result, they do not include the full spectrum of manifestations of a disease and may therefor be inadequate in the diagnosis of the individual patient (24). The ACR-criteria did not include a standard definition for making the initial diagnosis of WG, but described a group of patients where the diagnosis of WG was probable. An individual patient fulfilling the ACR criteria for WG may therefor have some other disease resembling WG (25). The systemic vasculitides used as controls in the ACR study also did not include the entity microscopic polyangiitis (MPA), a necrotizing small vessel vasculitis affecting arterioles, venules and capillaries resembling WG but without granulomatous inflammation (26). MPA may in many older studies have been included in the WG group or in the polyarterits nodosa group. The Chapel Hill consensus conference, on the other hand, has adopted an approach different from that of ACR and has classified the vasculitides according to the size of the vessels involved and constructed a standardised nomenclature system (22).

The epidemiology of WG is largely unknown. The incidence is low, diagnostic criteria vary and studies often come from tertiary referral hospitals introducing the possibility of selection bias. In the 1970`s the reported annual incidence was between 0.4 and 4 per million (27, 28). The estimates have increased over the last decade, which may be attributed to an increased awareness of the disease among clinicians, but also to the introduction of assays for anti-neutrophil cytoplasmic autoantibodies (ANCA) in 1985 (29, 30). Thus, a British study from 1995 reported an annual incidence of 8.5 per million (31). However, a true increase in incidence cannot be excluded.

The introduction of aggressive treatment with high doses of glucocorticosteroids combined with cyclophosphamide has improved the prospects for these patients. Fauci and co-workers induced remission in 93% out of 85 patients with WG and 88% were still alive after a mean follow up of 51 months (19). In the classical study by Hoffman and co-workers, 80% of the 158 patients were alive after eight years (21). Disease relapse is frequent however (32-34) and many patients also suffer from side effects of treatment including serious infections (35) and cancer (34, 36).

Antineutrophil cytoplasmic autoantibodies (ANCA)

The association between antibodies against neutrophils and WG was first reported in 1985 (29). Both neutrophils and monocytes, derived from the same lineage of precursor cells in the bone marrow, harbour the enzymes against which ANCA are directed, and both cell types have been reported to be present in several glomerular diseases, including crescentic glomerulonephritis (37, 38). Initially, the detection of ANCA was made by indirect immunofluorescence techniques (IIF), adding patient serum to ethanol fixed normal granulocytes. This results in either a granular, cytoplasmic ANCA (C-ANCA) or a perinuclear (P-ANCA) pattern (39). The perinuclear fluorescence pattern represents an artefact of ethanol fixation caused by the migration of positively charged constituents (like myeloperoxidase, elastase, lactoferrin) to the negatively charged nuclear membrane (40). Neutral or week cations (like proteinase 3, enolase) does not manifest this pattern, resulting in a cytoplasmic (C-ANCA) positivity. Solid phase assays to detect the nature of the ANCA specific antigens has later been developed and standarised (41), and are now very often used in combination with IIF.

The target antigen for ANCA in WG is most often proteinase 3 (PR3), a protein belonging to the serine proteinases present in azurophil granules of neutrophils and monocytes, but it may in as many as 24 % of the cases be myeloperoxidase (MPO) (42). The fact that the combination of P-ANCA pattern with anti-Pr3 positivity can appear, although less frequently, in patients with the other ANCA-associated small vessel vasculitides (42), is one of the reasons why the ANCA-tests alone should not be used as a diagnostic tool for WG in all clinical settings (43). The usefulness of a diagnostic test like ANCA relies highly on the prevalence of the disease in the population studied. In the clinical setting of rapidly progressive glomerulonephritis in adults, the combination of IIF-ANCA with a Pr3- ELISA gives a positive predictive value (PPV) of 98 % and a negative predictive value (NPV) of 80 % for the diagnosis pauci-immune crescentic glomerulonephritis (44).

Pathogenesis

The autoimmune etiopathogenesis of WG is still not fully elucidated (45), but a multitude of research focus on the triggering events that may be involved in the induction of autoimmunity. Inherited determinants may increase the risk (1, 46), but are probably not sufficient to induce the disease. Environmental factors such as infectious agents, especially *staphylococcus aureus* (47, 48), virus (49) and other factors such as silica exposure (50, 51), has been postulated to induce the autoimmune response resulting in WG.

A pathophysiological role of ANCA in WG has also been inferred (52). Priming of neutrophils by a variety of stimuli such as TNF-α, Il-1, IL-8, IF-γ, immune complexes and activated complement fragments, induces the translocation to the cell surfaces of several proteins from azurophilic granulae, among them Pr3 allowing ANCA to bind to them (53, 54). It has also recently been reported that during activation of Fc-γ receptors on the surface of neutrophils by ANCA, adhesion molecules are induced and maintained, making it possible for PMN to adhere to the endothelium and at this site to become finally activated (55, 56). A transient expression of PR3 on the endothelial cell surface (58) or the passive absorption onto the endothelial cell of systemically released PR3 (59), has also been proposed. This will allow ANCA to bind directly to the endothelial cells and thereby upregulate the expression of endothelial adhesion molecules. ANCA bound to Pr3 has been shown to enhance neutrophil oxidative burst and degranulation (53, 57). This chain of events can result in endothelial cell and vascular tissue injury, and has been put forward as a theory of the possible pathogenic role of ANCA in systemic vasculitis (52). The hypotheses on how ANCA mediate vessel injury is hampered by several uncertainties. Why was ANCA there in the first place, and why is it absent in the kidney biopsies in patients with WG and renal disease? ANCA is an immunoglobulin belonging to the IgG class, yet, in kidney biopsies of patients with WG a specific staining pattern for IgG or other immunoglobulins are usually absent (52, 60). In experimental animal models of vasculitis, glomerular deposits of complement and IgG are present in an early stage before the appearance of histopathological lesions, but absent at a later stage

probably because of rapid clearance by phagocytic cells (61). If similar stages of this process take place in humans, the early deposits may always be missed because of lack of clinical symptoms, the biopsy being taken at a later stage when lesions are present. This could be the reason for the paucity of immune deposits found in renal biopsies in patients with WG. The strongest argument for ANCA not being essential to WG however, is the observation that about one-third of patients with active but limited disease have been found to be ANCA-negative (62, 63). Even if they are not essential to WG, ANCA may still play an important role in enhancing the tissue injury in active disease (40).

Renal involvement in WG

The proportion of patients with renal involvement at disease presentation has varied between studies from less than 20% to 80%, but invariably increases to 80% to 94% during follow-up (21, 64, 65). The histopathological hallmarks of renal disease are the presence of segmental necrotizing glomerulonephritis with extracapillary proliferation and the absence or paucity of immunoglobulin or complement deposits (52, 60). The commonly held theory is that necrosis is the earliest glomerular sign in crescentic glomerulonephritis (66, 67). This leads to breaks in the glomerular basement membranes of the capillary tufts and extravasation of blood into Bowman`s space (66, 68). Activated macrophages proliferate and release growth factors causing glomerular epithelial cells to divide and accumulate and extracapillary proliferation (crescents) ensues. This process is probably also facilitated by the fibrin/fibronectin matrix forming in Bowman`s space partly caused by fibrinogen leakage from the glomerular tuft and partly by the pro-coagulant effects of macrophages (69, 70). There are, however, reports of patients with extracapillary proliferation without concurrent fibrinoid necrosis (71-73), findings which of course could be a question of representativity and of inadequate number of slides taken from each biopsy. The mechanisms of crescent formation in WG and the other vasculitides are still inadequately understood (74).

Interstitial leukocyte infiltration is another important feature of renal lesions in WG, and their intensity usually correlates with the severity of the glomerular lesions (75, 76). The infiltrates

often have periglomerular accentuation and concomitant breaks of Bowman`s capsule making it difficult to distinguish between glomerular and interstitial lesions (77). A granuloma-like reaction with accumulation of epitheloid cells and sometimes giant cells can be seen forming around a destructed glomeruli, a finding not specific for WG (21, 67). Only the infrequent occurrence in renal biopsies of interstitial necrotizing granulomatous inflammation without glomerular remnants is claimed to distinguish WG from renal lesion seen in other ANCA-associated small vessel vasculitides (microscopic polyangiitis, renal limited vasculitis and Churg Strauss disease) (52). Doubt has been put forward however, as to whether renal granulomas in any form should be regarded as typical for WG (78).

Scarring in the glomerulus and in the interstitial compartment has been shown to have a detrimental effect on outcome in many forms of progressive renal disease (69, 79, 80). The importance of the integrity of Bowman`s capsule in the development of glomerular and interstitial fibrosis in renal vasculitic disease has been emphasised (81, 82). Disruption of Bowman`s capsule allows inflammatory mediators including cytokines and lipid factors to leak out of the glomerulus into the interstitial compartment (83). Chemotactic factors, some of the most important being fibronectin fragments synthesised by cells within the crescents, attract cells that accumulate in the periglomerular region and, if the holes in BC are large enough, migrate into the glomerulus (84, 85). These cells can include macrophages, T-cells and fibroblasts (69, 81, 82). Fibrobalst-like cells lay down the collagen which constitutes the fibrous scar (fibrous crescents), and ultimately seal the fate of that glomerulus (glomerular sclerosis). Data support the hypothesis that transforming growth factor-β may be an important mediator promoting collagen synthesis in renal glomerular and interstitial disease (86, 87).

Clinically active renal disease is manifested by hematuria and erythrocyte casts, proteinuria and frequently by elevated serum creatinine concentration (64, 88, 89) and end stage renal disease will eventually develop in 11% to 32 % (21, 34, 89). Plasma exchange (PE) has been introduced as an adjunctive treatment for patients with particularly severe renal involvement, but the benefit of

this approach is debated (90-92). The rationale for using PE is the close association between ANCA and WG as well as the other small vessel vasculitides (42, 44, 93, 94).

Apart from the underlying renal disease itself, several factors have been identified with the potential to perpetuate the decline in renal function. These include hypertension, proteinuria, hyperlipidaemia, hyperparathyroidism, and hyperfiltration in remaining glomeruli (95, 96). Clinical management of renal disease in WG should therefor include a strategy for minimising the influences of some of these factors, as well as a treatment of the disease itself.

Renal biopsy in WG

To systematically quantify the extent to which morphological parameters appear in the renal biopsy can be of help in defining the most valuable predictors for renal outcome. However, the value of renal biopsy in WG is debated. Some reports have found pathological features such as glomerular necrosis, glomerular sclerosis and the number of crescents to be of little help in predicting renal outcome (72, 97). Others however, claim that renal biopsy provides valuable information when evaluating the activity of renal vasculitis and the degree of renal involvement, and argue that it is important for the therapeutic management (80, 98, 99). Cellular crescents formed in the early phases of crescentic glomerulonephritis can develop into mainly fibrous crescents (100, 101) notorious as predictors for bad prognosis because of their irreversibility. Along with features of interstitial fibrosis, it is conceivable that this can be of importance in the decision on whether immunosuppressive treatment should be instituted, continued or discontinued in the individual patient. Hence, deciding on the type and degree of renal involvement is a motive for using kidney biopsy in clinical practice. Some authors even argue that all patients with WG should have a renal biopy wether they have renal function abnormalities or not (102). For scentific purposes however, it is a problem that most studies have been too small to be conclusive. There is also no general agreement as to which renal lesions are of importance, so a comparison between studies is difficult.

Primary Sjögren`s syndrome (PSS)

The first description of the clinical features of keratitis, dry mouth and salivary gland enlargement is generally credited Hadden, Leber and Mikulicz in the late 1800`s . Although several reports followed, it was not until 1933 that the Swedish ophthalmologist Henrik Sjögren described, in detail, clinical and histological findings in 19 women, 13 of whom had probable rheumatoid arthritis with dry mouth and dry eyes (103). Sjögren introduced the term "keratoconjunctivitis sicca" for this syndrome, which later was named after him. PSS is a chronic inflammatory disease characterised by lymphocyte mediated infiltration of exocrine glands, especially lachrymal and salivary glands. It is a systemic disease with manifestations from several organ systems such as lungs, kidneys, skin, blood vessels and muscles, and lymphomas appear in about 5% of the patients (104). Secondary Sjögren`s syndrome is seen in patients with auto-immune diseases such as rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus and others. In the absence of these the disease is classified as PSS.

Disease classification and epidemiology

Diagnosis and classification of PSS have been difficult and four different classification criteria were suggested in the 1980s (105-108). All these criteria had in common a high specificity but a low sensitivity, features that are not useful for epidemiologic surveys since patients selected this way probably only represent a subset of the entire population.Two of the proposed criteria (107, 108) also did not permit the division between primary and secondary Sjögren`s syndrome. This is important since they differ in clinical (109) and genetic features (110, 111). The new European criteria proposed in 1993 (112) has a higher sensitivity (93.5%) and a lower specificity (94.0%) for the diagnosis of PSS than the beforementioned criteria (Table 2).

Table 2. European classification criteria for Sjögren`s syndrome

1.Ocular symptoms
A positive response to at least 1 of the following 3 questions:
Have you had daily, persistent, troublesome dry eyes for more than 3 months?
Do you have a recurrent feeling of sand in the eyes?
Do you use tear substitutes more than 3 times a day?
2.Oral symptoms
A positive response to at least 1 of the following 3 questions:
Have you had a daily feeling of dry mouth for more than 3 months?
Have you had recurrent or persistently swollen salivary glands as an adult?
Do you frequently drink liquids to aid in swallowing dry foods?
3.Ocular signs
Objective evidence of ocular involvement determined on the basis of at least 1 of the following 2 tests:
Schirmer-I test (≤5 mm in 5 minutes). Rose bengal score (≥ 4according to the van Bijsterveld scoring system)
4.Histopathological features
Focus score ≥ 1 in minor salivary gland biopsy
5. Salivary gland involvement
Objective evidence of salivary gland involvement, determined on the basis of a positive result on at least 1 of the following 3 tests: Salivary scintigraphy. Parotid sialography. Unstimulated salivary flow (≤ 1.5 ml in 15 minutes)
6.Autoantibodies
Presence of at least one of the following serum autoantibodies:
Antibodies to Ro/SS-A or La/SS-B antigens. Antinuclear antibodies.
Rheumatoid factor

The presence of 4 out of 6 items gives a sensitivity of 93.5% and a specificity of 94%. (From reference 112)

According to these new criteria a patient with symptomatic dry eyes or dry mouth can be diagnosed as having PSS with only one objective test of glandular dysfunction. This is in contrast to the Amercican criteria (108) which require both a abnormal lower lip biopsy and evidence of autoantibodies in order to make the diagnosis. It is not surprising therefor, that the European classification criteria gives prevalence numbers more than six times higher than the American criteria (113).

The true prevalence of PSS in the general population is not been established. In a British geriatric population clinical Sjögren`s syndrome was found to have a prevalence of 3.3% (114), and in a Swedish study the prevalence was reported to be 2.7% in the age group 52-72 years (115). There is a male to female ratio of 1:9 (113).

Renal involvement in PSS

Complete or incomplete distal renal tubular acidosis (dRTA) is the most common manifestation of renal involvement in PSS (116, 117) and is reported to occur in 18.4 % (118) to 67% (117) of the patients. This considerable variation is probably due to the different classification criteria used in the studies as well as to a selection bias. Patients with dRTA have an inability to acidify their urine despite severe metabolic acidosis. The disorder results from an impairment of net secretion of H^+ in the distal nephron, which in turn gives rise to reduced $HCO3^-$ regeneration (119). The pathophysiology for this defect is most often an insufficiency of the H^+-ATPase of the α-intercalated cells in the collecting ducts. The defect has been demonstrated in patients with dRTA secondary to PSS (120, 121). Alternatively, the capacity of the pump may be intact, but the ability of the distal tubular epithelium to maintain a high hydrogen gradient between the tubular cells and the urine may be impaired, resulting in the back diffusion of hydrogen ions (*gradient defect*) (119). A third mechanism for impaired distal acidification is a reduced cortical Na^+ reabsorption, thereby diminishing the degree of luminal negativity and producing a *voltage-dependent defect* (122).

In complete dRTA urine pH is always above 5.5 and there is a normal anion gap metabolic acidosis. In incomplete dRTA urinary pH is also above 5.5 at all times, but there is no systemic metabolic acidosis. In complete dRTA patients are either hypo- or normokalemic. Hypokalemia in dRTA is partly related to the reduced hydrogen secretion, where potassium secretion must be enhanced in order to maintain electroneutrality as sodium is reabsorbed, but decreased proton pump activity in it self can also lead to impaired K^+ reabsorption resulting in hypokalemia (123, 124). A third mechanism relates to the fact that increased sodium delivery to the distal collecting duct and increased levels of circulating aldosteron seen in dRTA, leads to increased renal K^+ secretion (125).

Hyposthenuria, due to an abnormality in the urine concentration mechanism, is also seen in patients with PSS (116, 126).

Clinically distal renal tubular acidosis is mostly silent, but there is an increased tendency to stone formation, and some patients may develop nephrocalcinosis and even renal failure (13, 127). This predisposition for renal calcification results from a number of factors. Urinary Ca^{2+} can be high secondary to acidosis-induced bone mineral dissolution (128). This increase of urinary Ca^{2+} is made worse by the low intraluminal concentration of HCO_3^- in the distal nephron. Normally HCO_3^- acts to increase distal Ca^{2+} reabsorbtion. Systemic acidemia, lowering the urinary concentration of HCO_3, results in a reduced Ca^{2+} reabsorbtion and an augmented urinary Ca^{2+} excretion (129). The high urinary pH decreases the solubility of calcium phosphate complexes, further enhancing stone formation (125). Hypocitraturia is a frequent finding among patients with coexistent dRTA and nephrolithiasis (130, 131). Citrate is an inhibitor of the crystallisation of stone-forming calcium salts, and low urinary citrate levels is therefor an important risk factor for urolithiasis. During acidosis an increased mitochondrial oxidation of citrate facilitates citrate reabsorption into the proximal tubular cells resulting in hypocitraturia (132).

The histopathological renal lesions most often reported in PSS is interstitial nephritis (133, 134). The interstitial inflammation is mostly focal and often associated with slight to moderate tubular atrophy. The tubulo-interstitial changes have been shown to correlate significantly with glomerular filtration rate (134). Glomerular disease is rare and when it occurs it is often associated with mixed cryoglobulinemia (135).

Aims of the thesis

The main objective of the studies upon which this thesis is based, was to improve our understanding of renal involvement in the two inflammatory rheumatic diseases Wegener`s granulomatosis and primary Sjögren`s syndrome.

The following goals were established:

- To evaluate the clinical course of patients with WG and renal involvement treated in eight Norwegian hospitals between 1988 and 1998 with special reference to relapse rate, renal and patient survival and morbidity from serious infections. To try to find out whether selected clinical variables had any influence on disease activity and patient outcome (Paper I).

- To do a follow-up of patients with WG treated with plasma exchange, with special emphasis on the patients presenting with a need for dialysis (Paper II).

- To examine through standard light microscopy and immunohistochemistry the histopathological changes seen in renal biopsies from patients with WG and varying degrees of renal involvement. We also wanted to study possible correlations between morphological features and the severity of the renal disease at the time of the biopsy and the development of end stage renal failure (Paper III).

- To describe the glomerular and interstitial inflammatory cells in patients with WG and renal involvement, and also to identify cells participating in early fibrogenesis. We wanted to see whether any of these cell types were markers for the severity of the renal disease at the time of biopsy and if their presence had any bearing on renal prognosis (Paper IV).

- To evaluate the prevalence and the severity of renal involvement in primary Sjögren`s syndrome diagnosed according to the preliminary classification criteria proposed by The European Classification Criteria Group. Emphasis was put on renal tubular function. We also wanted to find out whether biochemical markers of renal tubular damage were useful tools in identifying patients with distal renal tubular acidosis (Paper V).

Patients and methods

Patients

Paper I

Patients with WG and active renal disease treated in eight hospitals in Norway between 1988 and 1998 were included in this study. Each of the eight hospitals treated all patients with WG in its catchment area comprising approximately 2.2 million inhabitants, or about 50% of the total population of Norway.

All hospitals had an electronically based file for patient diagnosis, and records from these files were used to find patients with the diagnosis of WG (ICD-9 no 446.4). The information was then validated to confirm the diagnosis. The review covered all medical information available for each patient, and included an interview with the physicians who treated the patients. Autopsy reports were also obtained when they were available.

The diagnosis of WG was based on the clinical criteria developed by the American College of Rheumatology with at least two of the following criteria fulfilled: Oral ulcers or nasal discharge, abnormal findings on chest radiograph (nodules, cavities or fixed infiltrates), abnormal urinary sediment (red cell casts or more than 5 red blood cells per high power field), or granulomatous inflammation on biopsy (20) (Table 1). As an additional requirement, patients without a granulomatous inflammation shown on biopsy had to be seropositive for ANCA. A clinical diagnosis of active renal disease was defined as signs of active urine sediment i.e. more than 5 erythrocytes per high-power field (magnification, x400), or erythrocyte or granular casts. A total of 108 patients were included in the study.

Paper II

Twenty-nine of the WG patients described in paper I receiving plasma exchange (PE) as adjunctive treatment in the initial therapy, was the basis for this study.

Paper III

For 95 of the 108 patients described in paper in I, a percutaneous renal biopsy had been performed. In one patient the biopsy only contained 2 glomeruli and was therefor excluded from evaluation, leaving biopsies from 94 patients as the basis for the study. Table 3 depicts some characteristics of the 13 patients in the study not having a renal biopsy taken and, if possible, the reasons given for not doing a biopsy.

Table 3. Patient characteristics in 13 patients who did not have a renal biopsy taken at study start

Age	Sex	Serum creatinine*	Reason given for not doing a renal biopsy
72	M	Dialysis	Critically ill patient
26	F	102	Normal serum creatinine
67	M	Dialysis	Receiving heparin
72	M	77	Normal serum creatinine
76	M	72	Normal serum creatinine
72	F	490	Unknown
70	M	68	Normal serum creatinine
52	M	899	Unknown
70	M	248	Unknown
79	M	438	Considered unnecessary/age
68	F	159	Unknown
49	M	70	Normal serum creatinine
21	M	153	Unknown

M, male; F, female *Ref.values 60-120µmol/l

Paper IV

For 61 of the patients in paper III, additional paraffin embedded material was available for immunostaining. The analysis of these biopsies constituted the basis for this paper.

Paper V

Since 1992 patients with primary Sjögren`s syndrome living in the county of Hordaland have been registered consecutively at Haukeland University Hospital. The diagnosis of primary

Sjögren`s syndrome is established using the criteria proposed by The European Classification Criteria Group (112). All together the group contained 100 patients. Seventy of these patients who lived in and around the city of Bergen and therefor were able to reach the hospital within an hour or less, were invited to take part in the present study. Sixty-two patients (88.6 %) responded and were included.

Evaluation of disease activity (paper I, II, III, IV)

Disease activity of WG was defined as the presence of any of the following: 1) typical histologic abnormalities seen on biopsy of a clinically involved organ, 2) progression of upper or lower airway or ocular disease in the absence of infection or other illness, 3) progressive renal functional impairment as determined by active urinary sediment including red blood cell casts, 4) progressive polyneuropathy, nonvasculitic causes having been ruled out. 5) If a patient had none of the above but had an elevated erythrocyte sedimentation rate, constitutional symptoms, fever, or arthralgias/myalgias not related to identifiable nonvasculitic processes, the patient was also considered to have an active disease. (88)

Complete remission was defined as a state with no sign of active vasculitic disease and complete resolution of pulmonary infiltrates, improvement of renal function and resolution of extrarenal manifestation of vasculitis. The term partial remission was defined as a clear-cut suppression of the progression of disease activity with stabilisation of renal abnormalities, both functional and urinary findings, and partial resolution of pulmonary infiltrates. There should be no further worsening of other organ system disease activity, and there should be a progression towards improvement . Disease relapse was defined as the occurrence of any of the items 1-5 after having reached complete or partial remission.

Laboratory analysis (paper I, II, III, IV)

Blood tests

Routine laboratory tests were analysed at the departments of clinical chemistry at each hospital. ANCA were defined as either cytoplasmic (C-ANCA) or perinuclear (P-ANCA) and determined by indirect immunofluorescence microscopy (29). Atypical ANCA were not reported.

Renal biopsies (paper III and IV)

One or two cores of renal tissue were obtained by a through-cut technique, and the material was fixed in 10% buffered formalin and embedded in paraffin. The material was then sent from the local hospitals to the Norwegian Kidney Register in Bergen, Norway. 3 μm thick sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) for light microscopy. Sections from the paraffin embedded material were also examined by immunohistochemistry (PAP-method) for deposits of IgG, IgA, IgM, C3 and C1q. The number of glomeruli with extracapillary proliferation (cellular- and fibrocellular crescents) and the number of glomeruli with necrosis were expressed as percentages of the number of non-sclerotic glomeruli. The number of globally sclerotic glomeruli and the number of normal glomeruli were expressed as percentages of the total number of glomeruli in the biopsies. Crescentic glomeruli with breaks in Bowman`s capsule were also noted. Interstitial oedema and signs of acute tubular damage (ATD) like necrosis, flattening and shedding of the tubular epithelial cells, were recorded as dichotomous data (+/–). Acute interstitial nephritis was defined as interstitial infiltration by mononuclear and/or polymorphonuclear leukocytes, focally or diffusely. Changes characterised by infiltrates of mononuclear leukocytes associated with tubular atrophy and interstitial fibrosis were recorded as chronic interstitial nephritis. Subacute interstitial nephritis was a mixture of acute and chronic morphological features. The term "nephron loss" was used to describe the degree if chronicity in the biopsies as a summary of the following features: the degree of interstitial fibrosis, the degree of tubular atrophy and the fraction of globally sclerosed glomeruli. Nephron loss was scored on a four-point scale in the following manner: 0 = 0%, 1 < 25

%, 2 = 25-50% and 3 > 50%. Vasculitis was defined as infiltration of inflammatory cells within and around the vessel wall of arteries, arterioles or capillaries with or without fibrinoid necrosis. Granulomatous inflammation was defined as an accumulation of epitheloid cells with or without multinucleated giant cells. The location was specified as interstitial, vascular or adjacent to glomeruli with crescents and/or necrosis. Fourteen patients had a second renal biopsy taken at a later date, which was examined and compared to the initial biopsy.

The immunohistochemistry studies done in paper IV was performed by using the following antibodies: The *monoclonal antibody CD68*, clone PG-M1 (DAKO code no. M 0876) detects an antigen mainly located on lysosomal membranes after antigen retrieval by heating in citrate buffer. In formalin fixed, paraffin embedded material, the antibody labels macrophages. It does not react with granulocytes. *Polyclonal rabbit anti-human CD3 antibody* from DAKO (code no. A 0452) is considered a highly specific marker for T – lymphocytes, reacting with the intracytoplasmatic portion of the CD3 molecule, which is a part of the T-cell receptor complex. No other cells, except for Purkinje cells, are known to express the CD3 antigen. The *mouse monoclonal antibody CD8*, clone 1 A5, (Novocastra NCL-CD8-295) detects the CD8 co-receptor for MHC class I molecules found on cytotoxic/suppressor T-lymphocytes and also on some natural killer (NK) cells. The DAKO *mouse monoclonal antibody CD20*, clone L 26 (code no. M 0755) is directed against a membrane antigen, probably a calcium channel, present on B-lymphocytes. It is specific for B-lymphocytes and does not react with other hematopoietic cells. *Anti-human smooth muscle actin* clone 1A 4 (DAKO code no. M 0857), is a monoclonal mouse antibody reacting with the α-smooth muscle isoform of actin in smooth muscle cells, myoepitelial cells, pericytes and myofibroblasts. The *rabbit anti-human Ki-67 antibody* (DAKO code no. A0047), is an affinity-isolated antibody that has been shown to work on formalin fixed, paraffin embedded sections. It reacts with a nuclear antigen in proliferating cells after antigen retrieval by heating the tissue in citrate buffer. *Mouse anti-swine vimentin monoclonal antibody* (DAKO code no. M0725), marks intermediate filament

proteins mainly in cells of mesenchymal origin. It is also expressed in regenerating tubular epithelial cells.

Laboratory analysis (Paper V)

Histological analysis

A biopsy from the minor salivary gland of the lower lip was performed in 53 of the 62 patients at the time of the diagnosis, and evaluated with focus scoring according to the method described by Greenspan (136).

Blood analysis

A capillary blood sample was drawn for the determination of pH, standard bicarbonate and base excess (Ciba-Corning 865, Medfield, USA), The procedure was completed within 20 minutes and the specimen was kept on ice until the analyses were done. From venous blood, serum sodium, potassium, chloride, phosphate, creatinine and urate was measured by autoanalyser techniques (Technicon Chemicals 1, USA). Serum β_2-microglobulin was analysed by immunfluorescence (Abbot IMX, Chicago, USA), and ionised calcium with the use of an ioneselective electrode (Ciga-Corning 865, Medfield, USA). ANA was analysed with an indirect immunfluorescence technique with the use of HEp-2 cells, and antibodies against SS-A and SS-B was analysed by ELISA technique. An agglutination test was employed for detection of rheumatoid factor (MonoRheuma-test Skien, Norway), and nephelometry was used for quantification of IgG (Boehringer-Nephelometer). Sera were screened for cryoglobulin after 24 hours at 4°C.

Urine analyses

A sample of the 24-h urine was analysed for calcium, creatinine, sodium, potassium and chloride using an autoanalyser (Technicon Chem 1, US). The analyses were done on the day the urine collection was completed and immediately after the patient had arrived at the hospital. A

sample for urine cultivation was also taken at that time, and urine glucose concentration was measured with a semi-quantitative method (Combur-10-test, Meditron jr, Boehringer-Mannheim, Germany). Urine albumin was determined by nephelometry (Boeringer Nephelometer, Germany). Sulphuric acid was added to the urine until a pH of approximately 3 was reached before analyses of calcium were done.

A specimen of the 24-h urine and of the spot urine was kept frozen at minus 20 degrees centigrade until it was analysed for citrate, N-acetyl-beta-glucosaminidase (NAG), alkaline phosphatase (ALP), kallikrein, β_2-microglobulin, and creatinine. The analyses were performed with a Cobas Mira analyzer (Cobas Instruments, Roche Diagnostic Systems, Basel, Switzerland) at 37 ^{0}C. Citrate was analysed by an enzymatic method (137) and NAG by a colorimetric method (Boehringer Mannheim, Germany), based upon the release of 3-cresolsulfonphtalein from 3-cresol-sulfonphtaleinyl-N-acetyl-β-D-glucosaminide at 600 nm as an end point analysis. ALP was analysed colorimetrically at pH 9.8 and 405 nm by a kinetic method as *p*-nitrophenol liberated from *p*-nitrophenyl phosphate (Boehringer Mannheim, Germany). Kallikrein was measured spectrophotometrically using the tripeptide H-D-Val-Leu-Arg pNA as substrate(AB Kabi, Mølndal, Sweden) (138). β_2-microglobulin was determined by a commercial RIA kit (Pharmacia & Upjohn, Uppsala, Sweden), and creatinine by a modification of the Jaffe reaction (Beckman creatinine analyser Model II, Backman, Fullerton CA).

Reference values

Reference values for the blood analysis were supplied by the laboratory (Haukeland University Hospital, Laboratory for Clinical Biochemistry, Bergen, Norway).

Citrate and kallikrein in 24-h urine was expressed as mmol/24-h and units/24-h, respectively and β_2-microglobulin as μg/mmol creatinine. For the enzymes and for citrate in spot urine, the results were expressed as units/mmol creatinine. Median values, 2.5 and 97.5 percentile for citrate in normal controls were 3.09 (1.24 - 5.67) mmol in 24-h urine and 0.22 (0.10 and 0.50) mmol/mmol

creatinine in spot urine. For kallikrein in 24-h urine reference range was 14 –201 Ux24-h x 10^{-2}. Reference values for NAG, ALP and β_2-microglobulin, taken into consideration the length of time the urine had been frozen, were provided by the laboratory (Rikshospitalet, Oslo; Norway) (139).

Calculated values

Fractional sodium excretion was calculated as follows: (U-Na x S-creatinine/ U-creatinine x S-Na) x 100. Normal values<2%. As a marker for glomerular filtration rate, creatinine clearance was calculated and normalised to 1,73m^2 body surface area. Reference values were adjusted for age (140).

Renal concentration capacity

As a test for renal concentration capacity the patients received 40μg of 1-desamino-8-D-arginine-vasopressin (DDAVP) intranasally at 8 a.m. They voided immediately thereafter, and four hours later they voided again and urine osmolality was measured by freezing point depression (Fiske, Massachusets, USA). The patients were allowed to drink no more than 150 ml of fluid from the DDAVP was given until urine was voided. Reference values were adjusted for age (141).

Urine acidification

For patients with a fasting urine pH below 5.5 a normal acidification capacity was assumed. A diagnosis of complete distal renal tubular acidosis (dRTA) was made if patients had urinary pH above 5.5 and a metabolic acidosis (142).

For the remaining patients a short duration acidification test was performed on a later day using ammonium chloride loading (143). If urine pH decreased below 5,5 at any time the patients were assumed not to have a dRTA. If the urinary pH constantly was above 5.5, a diagnosis of incomplete dRTA was made.

Statistical analysis

Paper I and II

Relapse-free survival, renal (ESRD-free) survival and patient survival were described with the Kaplan-Meier method. The Mann-Whitney U-test was used to compare continuous variables, and χ^2 test was used to compare categorical variables between groups. The mortality risk ratio (MRR) of the 108 patients was calculated in a Cox proportional hazard model using all residents of Nord-Trøndelag county, age 20+years in 1984 as controls (n = 87.285). The analysis was controlled for age and gender. A MRR of 1 indicates that the observed number of deaths equals the expected number. Cox`s proportional hazards regression analysis was used to investigate whether selected variables predicted renal and patient survival, relapse and the occurrence of infections requiring hospitalisation. Only patients surviving the acute phase of the disease (3 months after inclusion) were considered to be at risk of developing relapse or end stage renal disease.

Paper III and IV

Spearman`s rank correlation coefficient (ρ) was used to test a possible association between morphological variables expressed on a continuos scale and serum creatinine. A non-parametric one way analysis of variance (Kruskal-Wallis test) was employed to evaluate the association between serum creatinine and morphological variables when these were expressed on a four-point scale. A Mann-Whitney U-test was used when the findings were expressed as dichotomous values. Multiple linear regression was used to see if selected histological variables had an independent influence on serum creatinine, and with Cox`s proportional hazard model we investigated whether histological features were significantly associated with the development of end stage renal disease (ESRD). Wilcoxon matched pairs signed rank sum test was used to make paired comparisons between morphological features when a follow-up biopsy was performed.

Paper V

The Mann-Whitney U-test was employed to compare continuous variables and Fisher`s exact test to compare categorical variables between groups. Pearson's correlation coefficient was used to measure the degree of association between continuous variables. The positive predictive value (PPV) and the negative predictive value (NPV) of the tests that were used to identify patients with dRTA , were calculated as follows: PPV = (True positives/true positives+false positives) x 100. NPV= (True negatives/true negatives+false negatives) x 100.

Significance (α) values

As some of the calculations in paper III and IV involved multiple comparisons using the same outcome variable (serum creatinine), the level of significance in these analyses were adjusted according to the Bonferroni method (144). For the rest of the analyses in papers I through V, the significance was defined as $P < 0.05$. All tests were two-tailed. The statistical analyses were done with the SPSS version 9.0 and 10.0 software package.

Ethical considerations

The patients had all given their written consent, and the studies were approved by the local Ethical Review Committee. The information located at the Norwegian Kidney Register was approved and licensed by The Norwegian Data Inspectorate.

Summary of results

Paper I

Median follow-up for the 108 patients was 41.5 months. Twenty-two patients (20.4%) were admitted with a need for dialysis. C-ANCA was positive in 87.6% and P-ANCA in 8.2% of the patients. Complete remission was obtained in 81.5% after a median of 4 months, and 54.7 % relapsed after a median of 22.5 months. Two and five year renal survival was 86% and 75% respectively, and 22.8% of the patients living longer than three months developed ESRD. Two and five year patient survival was 88% and 74% respectively, and 24.1 % died after a median of 31 months. 46.2 % percent of the deaths could be related to WG and 23.1% to side-effects of the treatment. The cumulative mortality was 3.8 times higher than expected. Thirty-one percent of the patients were hospitalised for serious infections during follow-up, the most frequent infective agent being *Pneumocystis carinii*. The relative risk of relapse increased with the use of intravenous pulse cyclophosphamide compared to daily oral cyclophosphamide. Initial renal function predicted renal survival and low serum albumin and high age at treatment start increased the mortality risk. Old age increased the risk of having an infection.

Paper II

The 29 patients receiving PE were younger, with a higher fraction presenting with a need for dialysis (17/29) and with more organ systems involved by WG than the patients receiving only immunosuppressive therapy. The review of the renal biopsies also revealed a significantly higher fraction of glomeruli with extracapillary proliferation and a lower fraction of normal glomeruli in the PE group.

ESRD evolved in 37.9 % of the patients. Two and five year renal survival was 74 and 54 % respectively. One month after therapy was started, seven out of 14 patients (50%) alive in the PE group had been able to discontinue dialysis compared to one out of five (20%) in the

immunosuppression only group (NS). At the end of follow-up six (42.9%) of the 14 patients alive in the PE group had independent renal function but none had normal serum creatinine concentration. When a Cox proportional hazard model was employed adjusting for the serum creatinine and the number of organs effected by WG, there was no difference in the development of ESRD between the patients treated with PE and those treated with immunosuppression alone. No serious side-effects from PE were detected.

Paper III

In renal biopsies from the 94 patients segmental necrotizing glomerulonephritis and extracapillary proliferation were present in 85.1% and 91.5% respectively. In seven patients (7.4%) with normal serum creatinine and urinary protein excretion <0.5 g/day, all had crescents and six had segmental glomerular necrosis. Breaks in Bowman`s capsule was observed in 74.7% of patients with crescents. One patient had an interstitial granuloma. Weak granular staining for immunoglobulins or complement was demonstrated in 28.0% of the patients, the most frequent being IgM and C3 respectively. Distinct mesangial IgA positivity was seen in two patients. Serum creatinine at biopsy correlated significantly with the percentage of glomeruli with crescents (ρ=0.52 P=0.0004), with necrosis (ρ=0.36 P=0.002) and with the percentage of normal glomeruli (ρ= -0.55 P=0.0003). There was also a significant correlation between serum creatinine and interstitial inflammation and acute tubular necrosis. On a multivariate analysis only the percentage of normal glomeruli was significantly associated with renal function and development of ESRD. In 14 second biopsies after a mean of 41.2(±26) months, chronicity scores had increased significantly in 13 patients in spite of full immunosuppressive treatment.

Paper IV

Inflammatory cells

The majority of the inflammatory cells were $CD68^+$ (macrophages). More than 2/3 of the lymphocytes were $CD8^+$ cells (cytotoxic T-cells). There were an equal number of $CD3^+$ and $CD68^+$ cells distributed in the interstitial areas. $CD68^+$, $CD3^+$ and $CD8^+$ cells were abundant in the periglomerular areas especially in glomeruli with crescents. No intraglomerular $CD20^+$ cells (B-lymphocytes) were found. The number of macrophages both in the glomeruli and in the interstitium correlated significantly with serum creatinine at the time of biopsy but not with creatinine after one year. The number of interstitial $CD3^+$ and CD8 cells correlated also with serum creatinine at biopsy but not after one year. The number of interstitial $CD3^+$, $CD8^+$ and $CD68^+$ cells correlated significantly with the fraction of glomeruli with rupture of Bowman`s capsule.

α-SMA and vimentin

The staining for α-SMA in the glomerulus in patients with WG was weak and not significantly different from tissue obtained in controls. Some, but not all crescents were positive for α-SMA, especially where breaks in Bowman`s capsule was noted. Pericapsular staining for α-SMA was prominent in all the WG patients, especially around glomeruli containing crescents. Serum creatinine at biopsy differed significantly among the three levels of interstitial α-SMA staining After one year patients belonging to the group with the strongest staining of α-SMA had significantly higher serum creatinine than patients in the two other groups. Serum creatinine at biopsy was also different in the three levels of tubular vimentin staining, but no difference was observed after one year. Tubular vimentin staining was significantly higher in patients with, than in patients without, signs of acute tubular damage (ATD).

Paper V

Age adjusted creatinine clearance was reduced in 13(21%), and 21% had reduced age adjusted maximum urine concentration capacity. Albumin excretion was more than 30 µg/min in only one patient (1.6 %). Four patients (6.5%) had complete and three patients (4.8%) had incomplete distal renal tubular acidosis (dRTA). Four of the seven patients (57.1%) with complete or incomplete dRTA had reduced creatinine clearance and five (71.4%) had reduced maximum urine concentration capacity. All patients with dRTA had positive ANA. Six of the seven patients (85.7 %) with dRTA tested positive for antibodies towards SSA or SSB antigens compared to 14 out of 55 (25.5 %) patients without dRTA (P=0.003). The average number of inflammatory foci in biopsies taken from salivary glands of the lower lip was 4.7 in patients with, compared to 1.8 in patients without dRTA (P=0.002). The ratio citrate/creatinine in spot urine was below the 2.5 percentile in all patients with complete or incomplete dRTA, and the NPV was 100% and the PPV was 90% when citrate in spot urine was used to identify patients with dRTA.

General discussion

The aim of this thesis was to evaluate different aspects of renal involvement in the two inflammatory rheumatic diseases WG and primary Sjögren`s syndrome. Paper I is a retrospective analysis of the clinical course of 108 patients with WG where the renal disease was diagnosed and treated in the years 1988 to 1998. In paper II we evaluate the clinical course of 29 patients with WG treated with plasma exchange. In paper III we report on a study of 94 patients from the same cohort of WG patients where a kidney biopsy was performed and evaluated using standard light microscopy and immunohistochemistry. In paper IV we did a study using immunohistochemistry on paraffin embedded renal biopsies to identify inflammatory cells and also identify cells taking part in early fibrogenesis. Paper V is a study of renal glomerular and tubular involvement in 62 patients with primary Sjögren`s syndrome classified with the new European classification criteria.

Clinical course in patients with WG and renal disease

In the 108 patients with WG and active renal disease, complete remission was achieved in 81.5% of the patients following induction therapy and 55% experienced at least one relapse, occurring after a median of 22.5 months. The remission rate was somewhat lower than reported in the study by Fauci and co-workers (19), but comparable to results of studies where only patients with renal disease were included (34, 145). A high proportion of our patients relapsed and relapses came earlier than reported by others (145, 146). Some studies indicate an increased risk of relapse in small vessel vasculitis where patients have antibodies directed against aPR3 rather than against aMPO (33, 147). The presence of these antibodies were not tested in all our patients, but a majority were c-ANCA positive, which makes it conceivable that aPR3 was more frequent than aMPA.

Intravenous cyclophosphamide given in pulses increased the likelihood of relapse compared to daily oral administration after adjusting for age, serum creatinine and for the number of organs affected. The exact treatment schedules varied, but many patients experienced a relapsed when the dose-interval increased. Intermittent intravenous treatment with cyclophosphamide has been

introduced with the intention of reducing side effects from high cumulative doses of the drug, but firm evidence that this can be achieved without compromising remission and relapse rates are lacking. Our results may suggest that fear of side effects could have led to suboptimal cyclophosphamide dose intensity in the intravenous treated group. A higher risk for relapse in patients treated with intravenous pulse- compared to daily oral cyclophosphamide has also been reported in a recent prospective study (35). At the time of relapse the majority of our patients did not receive cytotoxic agents at all. In the study by Fauci, where only 29% of the patients had relapsed after a mean follow up of 51 months, the treatment was more prolonged with a mean duration of therapy of 35 months (19). These observations can constitute an argument for prolonged therapy including a cytotoxic agent in order to maintain remission.

Renal functional status at entry was a predictor of renal outcome, and of the patients who were initially on dialysis, 45.5 % developed ESRD. Results from ours and other studies (80, 145, 148, 149) indicate that a majority of patients on dialysis will profit from treatment and regain a substantial amount of renal function, although usually not total normalisation.

The cumulative mortality in our study was 3.8 times higher than expected from the reference population and approximately 70 % of the deaths were related to WG or side effects of the treatment. In a North American study published in 1996 the overall mortality was 4.7 times higher than expected (3), a number comparable to ours. All our patients had renal involvement, and patients with generalised disease are known to have shorter life expectancies than patients with limited disease (2, 3, 89). A substantial early mortality (27% of all deaths) is an observation also reported by others (3, 146, 147). This is in contrast to the lower mortality associated with relapse, probably because most patients at that time are under closer surveillance so that adequate treatment can be instituted early. We found patient survival to be influenced by age and serum albumin. The reason for the effect of serum albumin on survival could be that it is a parameter influenced both by the degree of systemic inflammatory response and by renal protein loss.

About one third of the patients received in-hospital treatment for a serious infection during the study period and old patients were at highest risk. *Pneumocystis carinii* was the most frequent infective agent and two of the patients with PCP died. A similar frequency of PCP has been noted in other studies (35) and lymphopenic patients, mainly lacking CD4 T-cells, seem to be at particular risk (150, 151). This suggests that prophylactic trimethoprim-sulphamethoxasole should be used during the time of maximum immunosuppression and especially when a cytotoxic agent is combined with high doses of corticosteroids (152, 153). The frequency of malignant disease was low in our study, probably because the observation time was relatively short. Others have reported an increased incidence of malignant disease in patients treated for vasculitis (21, 34), and the relationship between long-term use of cyclophosphamide and the development of bladder cancer (36) and leukemia and preleukemia (154), is well recognised.

Plasma exchange in WG

PE was employed in patients with serious renal involvement, both measured by serum creatinine concentration and by renal morphology. Our observational study was not designed to resolve the question of whether PE significantly improves renal outcome in patients with WG, but it points to the unfavourable effect on renal prognosis of patients presenting with severely impaired renal function, even if PE is included in the therapy. Five-year renal survival in this cohort was only 54%. Balow (155) reported a renal and patients survival of 55 % at five years in patients with WG and rapidly progressive glomerulonephritis treated with CS and CYC. In a series of 31 patients with severe RPGN where 68 % were dialysis dependent at presentation, the three year renal survival was 78 % (92) a number not very different from the two year renal survival of 74% in the PE group in our study. The authors concluded that there was no difference in renal outcome whether the patients received immunosuppression alone or PE was added. Recently a retrospective study was published reporting on the follow-up of 73 patients with ANCA associated crescentic glomerulonephritis with creatinine > 500 μmol/l or dialysis dependent (55/73), treated with oral CYC, oral CS and at least

five plasma exchanges (156). In this group of patients which was comparable to the PE-treated group in our study, they were able to show a remarkable 83 % five year renal survival. A total of 21 patients (28%) died however, a number comparable to ours. Information on the last serum creatinine before death in these patients is not given. Even though one randomised study has shown a benefit of PE in dialysis dependent patients with ANCA associated small vessel vasculitis (91), most studies has failed to show this (90, 92, 157).

Although the results at the end of follow-up in our study is not significantly different from other series, some studies using PE have shown better results than we have in the speed of response in the early course of the disease (91, 157). The reason for this is unknown. Patient selection can be different, and some studies may have used more daily exchanges in the beginning of the treatment or a higher total number of exchanges than we have done (90, 158). Whether this can result in patients getting off dialysis earlier is possible, but remains to be proven.

As there are no or very few antibodies in renal biopsies taken from these patients (52, 60), PE may be done too late in the course of the disease to have any effect on antibody clearance. However, other beneficial effects of extracorporal treatment could be possible, such as removal of inflammatory mediators or modulation of cellular responses (159).

Renal histopathology in WG

The morphological studies displays the wide range of changes seen in renal biopsies from patients with Wegener`s granulomatosis. It also points to the fact that even in the absence of obvious renal impairment, segmental necotizing glomerulonephritis and severe extracapillary proliferation can exist. Patients with necrosis and crescents all had rather extensive hematuria, suggesting that destructive inflammation in glomerular capillaries with bleeding into Bowman`s space could be one of the early events in the development of glomerulonephritis in WG. Our findings indicate that morphological criteria might be a better method of defining renal involvement in vasculitis than renal functional measurements, and could be of importance in deciding whether or

not aggressive treatment should be initiated. The prevalence of immune deposits was higher in our study than in most earlier series of patients with ANCA associated vasculitis (160-162), but in a recent report from the European multicenter trials (EUVAS) the prevalence compared well with ours (163). Except in two patients with diffuse granular deposits of IgA, the immune staining was of low intensity and focally distributed and probably a consequence of passive trapping in the mesangium.

Using univariate analysis we have found a positive correlation between serum creatinine and the fraction of active glomerular lesions (crescents, necrosis) and a negative correlation between serum creatinine and the fraction of normal glomeruli. These relationships were best described in a non-linear regression model (exponential). A recently published study did not find active glomerular lesion to be significantly correlated to serum creatinine (163), but only 88 of their 154 patients represented WG, the others being microscopic polyangiitis (MPA), idiopathic rapidly progressive glomerulonephritis (iRPGN) and Churg-Strauss syndrome. Renal biopsies in WG may be characterized by active lesions compared to MPA and iRPGN were chronicity is more predominant (164, 165). This could indicate that different morphologic features correlate to renal function in WG compared to the other clinical entities. On a multiple regression analysis only the percentage of normal glomeruli had a significant independent influence on serum creatinine.

There was a relatively short time span from the first symptoms related to WG and renal biopsy in our patients. Signs of chronicity expressed in the term nephron loss (tubular atrophy, glomerular sclerosis, and interstitial fibrosis) were therefore rather uncommon in the biopsies and this could be the reason for the lack of prognostic impact of these indices. There was however, a striking increase in these features in 13 of the 14 repeat biopsies after a mean follow-up of three and a half years. All of the 14 patients were treated with corticosteroids and immunosuppressant drugs, but this obviously did not stop the development of chronic renal failure in the majority of the patients, seven of which had one or more relapse and one had a smoldering disease.

Inflammatory cells and markers of repair and fibrosis

Intraglomerular leukocytes were mainly $CD68^+$ cells (macrophages) and to a lesser extent T-lymphocytes. The dominant lymphocyte subpopulation was cytotoxic ($CD8^+$) cells and there were no B-lymphocytes in the glomeruli. We found glomerular and interstitial macrophages and interstitial lymphocytes to be significantly correlated to glomerular renal function at the time of biopsy but not after one year.

The important role for macrophages in crescent formation has long been known (166). Macrophage presence and proliferation are prominent in rat crescentic glomerulonephritis, but not in normal rat kidney or in mild glomerular injury induced by hypercholesterolemia (167). In man, glomerular macrophage infiltration in ANCA associated glomerulonephritis has been shown to be extensive, and found exclusively in areas of necrosis, where it is co-localised with adhesion molecules (ICAM-1 and VCAM-1) (168). Macrophages are established cytotoxic effector cells in type IV immune responses and can upon activation release oxygen radicals and lysosomal enzymes that damage adjacent cells (169). They can also synthesise and release a variety of cytokines and growth factors that, in turn, attract more infiltrating immune cells and activate resident cells, thereby amplifying the tissue injury (167, 170). Despite the highly significant correlation between the number of interstitial and glomerular macrophages and serum creatinine at the time of biopsy found in the present study, the macrophage induced acute renal impairment was largely reversible by treatment, as no correlation to longterm renal outcome could be shown.

There is substantial experimental evidence that T cells can induce acute glomerular injury even in the absence of glomerular antibody deposition (171, 172). The importance of T-cells in crescent formation was demonstrated in a model of crescentic glomerulonephritis in WKY rats where blocking of the CD28/B7 co-stimulatory signal for T-cell activation led to a marked reduction in crescent formation, even when it was administered after the disease was established (173). Studies of human glomerulonephritis have also reported an association between T-cells accumulating in glomeruli and crescentic glomerulonephritis (174, 175). Patients with ANCA

associated vasculitis have been shown to have an increased expression of markers for T cell activation, regardless of disease phase or immunosuppressive therapy (176), and autoreactive PR3 specific T-cells are present in patients with WG (177).

Studies of crescentic glomerulonephritis in rats indicate a crucial role for $CD8^+$ cells in crescent formation (178, 179). Activation of lymphocytes, including $CD8^+$ cells, as shown in systemic vasculitis (176), leads to enhanced production of cytokines and chemokines which in turn increases the expression of adhesion molecules on endothelial cells and circulating leukocytes (77). This results in accumulation of macrophages and activated leukocytes in the glomerular tuft eventually inducing necrotizing capillary damage (180).

We have found renal glomerular function at the time of biopsy and after one year to be significantly poorer with increasing intensity of interstitial α-SMA staining. α-SMA is expressed by myofibroblasts that are cells sharing features both of smooth muscle cells and fibroblasts (181). Transforming growth factor-beta (TGF-β) has been found to be involved in the activation and differentiation of myofibroblasts (182) and collagen synthesis in renal cortex in rats was highly correlated to increased activity of TGF-β (87, 183). Our study can indicate that infiltration by myofibroblast is an early sign of the fibrotic process occurring in the kidneys in our cohort of patients.

There was a significant positive correlation between renal tubular vimentin expression and serum creatinine at the time of biopsy, but not after one year. Vimentin expression was strongest in patients with signs of acute tubular damage. These observations suggest that vimentin is associated with the acute tubular injury often seen in renal vasculitis, and is in keeping with a report of increased vimentin expression by regenerating rat proximal tubular cells in response to acute injury (184). Thus, transient vimentin expression may be regarded as a marker of regenerating and proliferating tubular cells, whereas persistent expression can be a sign of chronic renal damage. Vimentin analysis in repeat biopsies could be a method for the testing of this hypothesis.

Renal involvement in PSS

Complete or incomplete distal renal tubular acidosis (dRTA) was confirmed in 11.3% of our 62 patients with primary Sjögren`s syndrome. Earlier studies (117, 118) have reported higher frequencies, and in a major textbook of rheumatology (12), 35% is said to have an abnormal urine acidification test. In the present study we have employed the new European classification criteria with a higher sensitivity but a lower specificity for primary Sjögren`s syndrome than criteria used in previous studies (112). According to these criteria, patients can be classified as having primary Sjögren`s syndrome even without autoantibodies or inflammatory foci on salivary gland biopsies. It is therefor of interest that all our patients with dRTA had positive ANA, 85.7 % tested positive to either SSA or SSB, and the number of focus scores were significantly higher than for patients without dRTA. The association between hypergammaglobulinmia and dRTA has been noted earlier (116), and in our study there was a tendency toward higher IgG in patients with dRTA, but the difference was not significant. The study indicates that patients with dRTA represents a cohort within the primary Sjögren`s syndrome population with more extensive immunologic and histologic involvement.

Reduced creatinine clearance was found in 21% of our patients. In a retrospective study by Vitali (11) two percent of the 104 patients had a creatinine clearance less than 60 ml/min. This is in striking contrast to a recent Swedish paper were 33 % was found to have reduced ^{51}Cr-EDTA clearance (185). The measurement of creatinine clearance as an estimate of GFR introduces the uncertainty of 24-hour urine collection. If less than the total urine volume is collected, the calculated GFR will be underestimated. Therefor, reduction of GFR found in 21% of our patients could represent an overestimate. As none of the patients in the present study had overt proteinuria and only one had microalbuminuria, it is reasonable to conclude that the cause of the reduced GFR is not primarily glomerular but rather secondary to tubulointerstitial dysfunction.

An abnormality of the urine concentration mechanism was found in 21 % of all patients and in five out of the seven patients with dRTA. The results in earlier studies have shown considerable

variation, from 16 % (186) to 58 % (126) of patients. The methods used have varied, and the results have not always been adjusted for age. It seems obvious from our and other studies that hyposthenuria can be seen in primary Sjögren`s syndrome even in patients without concomitant acidification defects.

All our patients with complete or incomplete dRTA had citrate values below the 2.5 percentile of normal controls, both in 24-h urine and in spot urine. Among our patients with primary Sjögren`s syndrome normal values of citrate in urine excluded the possibility of either complete or incomplete dRTA, but low values did include some false positives. This of course would be expected, as the cut-off value was the 2,5 percentile in normal controls.

β_2-miocroglobulin is a freely filterable protein which under normal circumstances is almost totally reabsorbed in the proximal tubule (187). Damage to this section of the nephron leads to increased recovery of β_2-microglobulin in the urine. In the face of elevated serum levels, the reabsorbtive capacity can be surpassed even in the normal tubule, resulting in increased urinary β_2-microglobulin. Only one patient had serum concentration above the upper limit however, so this cannot account for the high proportion of patients with increased excretion of β_2 – microglobulin in our material. Urinary NAG and ALP, both markers of proximal tubular damage, were also elevated in a considerable fraction of our patients. Both enzymes showed a negative correlation to creatinine clearance (results not shown) accounting for part of the increase. Eriksson and co-workers found elevated urinary NAG and α_1-microglobulin in 29% and 46% of patients with primary Sjögren`s syndrome in the absence of other clear evidence of proximal renal tubular damage (117). We can as now not fully explain the significance of these findings, and although urinary excretion of β_2-microglobulin, ALP and NAG were significantly higher in patients with than without dRTA, they were not especially helpful in identifying patients with this abnormality.

Limitation of the study

The methods used in the recruitment of patients are always crucial in clinical studies. In paper I, II, III and IV we used the ACR classification criteria for the diagnosis of WG (Table1). In the original study leading to the proposed criteria, the reference population consisted of patients with verified vasculitic diseases (20). The predictive value for WG cited in their publication will not pertain to patients without proven vasculitis. We therefor added to our inclusion criteria a requirement for either ANCA positivity or a biopsy showing granulomatous inflammation. A positive test for C or P-ANCA predicts a 96% probability that a patient has or shortly will develop a necrotizing vasculitis or crescentic glomerulonephritis (188). Still, we may have included some patients not having WG, probably patients with the diagnosis MPA.

As we used an electronically based coding system to select patients, we may have missed patients who were incorrectly diagnosed. We tried to get around this problem by interviewing doctors treating patients with vasculitis in each hospital and in that way include patients missed.

The retrospective design of the WG studies also represents a limiting factor. It makes it difficult to draw firm conclusion of the effect of intervention variables such as treatment modalities even after adjusting for possible confounding variables.

For primary Sjögren`s syndrome no uniformly accepted diagnostic criteria exists. The real prevalence of the disease is therefor unknown. The patients referred to our centre at Haukeland University Hospital came from general practitioners, ophthalmologists, rheumatologists, specialists in internal medicine and ENT-specialists. There is always a chance for a selection bias, as patients with only minor manifestations of the disease probably will be referred to a lesser extent than patients more seriously effected. The prevalence numbers of renal involvement in our study may therefor represent maximum estimates.

When we selected the 70 patients invited to take part in the study, we included patients who could reach the hospital by bus or taxi within an hour, as the entire study was done on an outpatient basis. As primary Sjögren`s syndrome is not a disabling disease, we don't expect this cohort of

patients to have significantly different disease manifestations than the total group. The demographic and clinical characteristics of the study group and the group of patients not included were compared and found to be similar (Results not shown).

Summary and conclusions

- More than half of the patients with WG experienced at least one relapse within 41.5 months. The mortality was 3.8 times higher than in a control population. The disease itself caused 46 % of the deaths and 23 % was caused by side effects of the treatment. Chronic renal failure developed in 23 % of the patients.
- About one third of the patients were hospitalised for serious infections during the treatment, *pneumocystis carinii* pneumonia being the most commonly reported cause.
- This study indicates that treatment with intravenous pulse cyclophosphamide increases the likelihood of relapse compared to daily oral treatment.
- The prevailing practice among hospitals participating in our study was to give PE to the most seriously ill patients. Although a majority of patients on dialysis profit from treatment, a normalisation of serum creatinine is seldomly obtained even if PE is used as adjunctive therapy
- The strongest predictor of renal function and of renal prognosis was the fraction of uninjured glomeruli in the renal biopsies. The correlation is week however, and although the initial renal biopsy gives valuable information on the degree of renal involvement in WG, it does not seem to be of substantial help in predicting the course of renal function in the individual patient.
- Even in the absence of obvious renal functional impairment, crescentic necrotizing glomerulonephritis may be present.
- A follow-up renal biopsy can be useful in revealing the degree of activity and chronicity and may be of importance for the choice of further therapy.
- Our study supports the suggestion that systemic vasculitis is a caused by type IV cellular immunity. Macrophages are the most numerous infiltrating cells in the glomeruli and $CD8^+$ cells were the dominant lymphocyte subpopulation in the glomeruli. Cells staining positive for α-smooth muscle actin (α-SMA) correlated inversely with renal outcome.

- The prevalence of dRTA in primary Sjögren`s syndrome was lower than previously reported and it was mainly found in patients with extensive immunologic and histologic involvement.

- Creatinine clearance was reduced in more patients with primary Sjögren`s syndrome than found in earlier studies, but clear evidence of glomerular disease was lacking.

- All patients with incomplete or complete dRTA had low values of urinary citrate both in spot urine and in 24-h urinary collections. Normal values of the citrate to creatinine ratio in spot urine seem to exclude the possibility of dRTA. In the event of low values, ammonium chloride loading is still necessary to exclude dRTA.

Consequences

A strategy for prolonging remission without inducing serious complications from the treatment must be an important objective for further prospective studies of WG. The efficacy of other therapeutic candidates such as azathioprine, methotrexate, mycophenolate mofetil, cyclosporin A, intravenous immunoglobulin or monoclonal antibodies against T-cells in generalised disease and the role of trimethoprim-sulphamethoxasole for localized WG needs to be elucidated. The current therapy with corticosteroids and cyclophosphamide may fail to downregulate the T-cell activation seen in WG. Blocking the T-activation by inhibiting cell surface proteins (B7, CD2) or inhibiting stimulatory cytokines (IL-1, IL-2, IFN-γ, IL-12), may be a strategy worth exploring in the future. Randomised controlled studies comparing pulse intravenous methylprednisolon and PE as additive treatment are also needed, and are under way. Suppression of collagen production by cells like myofibroblasts has been demonstrated with the use of agents blocking the effects of TGF-β (86). Until further studies are undertaken and concluded, care should be taken not to prolong the induction treatment with cyclophosphamide unnecessarily and probably substitute it with a less toxic regimen for remission therapy. Our results indicate that prophylactic trimethoprim-sulphamethoxasole should be used during the time of maximum immunosuppression and especially

when a cytotoxic agent is combined with high doses of corticosteroids. Because renal involvement was shown to be present in some patients with normal serum creatinine concentration, renal biopsy should be performed in any case where microscopic hematuria is present.

In patients with primary Sjögren`s syndrome, an initial screening for metabolic acidosis should be done and the citrate /creatinine ratio in spot urine calculated. If the citrate/creatinine ratio is low and the patient have had renal calculi or ultrasound shows nephrocalcinosis, treatment with potassium-citrate should probably be initiated.

Erratum

Paper I

p 614: section *Renal status at follow-up*, second paragraph, line 5.

The passage now reads: Twelve (52,2%) of the 23 patients developing ESRD regained renal function and were off dialysis after a mean of 35.8 (+/-31.9) days.

The passage should read: Twelve (54.5 %) of the 22 patients initially on dialysis regained renal function and were off dialysis after a mean of 35.8 (+/- 31.9) days.

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Paper I

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Paper IV

Wegener`s Granulomatosis: Inflammatory Cells and Markers of Repair and Fibrosis in Renal Biopsies - a Clinicopathological Study

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Running title:

Renal inflammatory cells in Wegener`s granulomatosis

Abstract

Objective: To quantitate inflammatory cells in renal biopsies from patients with Wegener`s granulomatosis (WG) and to identify cells participating in early fibrogenesis. We wanted to see whether these cells correlated with the severity of renal disease and if their presence had a bearing on renal prognosis. *Material and methods:* Sixty-one patients with WG who had a renal biopsy taken at the time of diagnosis were included in the study. Immunostaining with monoclonal antibodies toward macrophages (CD68), T- and B lymphocytes, α-smooth muscle actin (α-SMA) and vimentin was done. *Results:* The dominating intraglomerular leukocytes were macrophages (29.9 ± 15 cells/glomerular cross section) and to a lesser extent T-cells (2.57 ± 1.8 cells/glomerular cross section). No B-lymphocytes were detected in the glomeruli. More than 2/3 of the T-cells was $CD8^+$ (cytotoxic) cells. Macrophages and T-lymphocytes were equally distributed in the renal interstitium and were numerous around crescentic glomeruli. Glomerular and interstitial macrophages and interstitial T-cells correlated significantly with serum creatinine at the time of biopsy but not after one year. Serum creatinine at the time of biopsy and after one year differed significantly among the three levels of interstitial α-SMA staining. Serum creatinine at biopsy was highest when tubular vimentin staining was strongest, and tubular vimentin staining was strongest in patients with acute tubular damage. *Conclusions*: We have found evidence for a cellular type IV immune response in WG, with $CD8^+$ T lymphocytes and macrophages dominating the cellular infiltrate. The detection of interstitial α-SMA, probably staining myofibroblasts implicated in renal fibrogenesis, indicated low GFR one year after renal biopsy.

Key words: Wegener`s granulomatosis, renal biopsy, macrophages, lymphocytes, myofibroblasts, vimentin, renal function.

Introduction

Although treatment with high doses of corticosteroids combined with cyclophosphamide has brought significant improvement to the prognosis of patients with small vessel vasculitis (1), a substantial fraction will still develop end stage renal disease (2, 3). Initial impairment of glomerular filtration rate and severe proteinuria have in several studies been found to be the most powerful clinical predictors of subsequent renal detoriation (2-5). The prognostic value of kidney biopsy is debated, and even though some researchers have found standard light microscopy to provide valuable information as to the extent of renal involvement (6-8), the predictive value, especially of glomerular features in the early kidney biopsy, seems to be limited (9, 10).

The recent search for new therapies for patients with Wegener`s granulomatosis and the other small vessel vasculitides, has mostly aimed at reversing the inflammatory events driving the disease process (11-14). Identifying and understanding the immunopathological mechanisms of disease can be of help in the research for new therapeutic targets. Monocytes/macrophages and several subsets of T lymphocytes are implicated in the pathogenesis of the acute glomerular and interstitial disease, and evidence of a delayed type hypersensitivity (type IV) reaction has been found in glomerular crescent formation (15). The scarring process, eventually sealing the fate of the glomerulus and leading to interstitial fibrosis, is driven by prosclerotic forces, but the mediators involved in initiation and maintenance of this process are partly unknown. Cells with a myofibroblast phenotype seems, however, to play an important role (16). These cells share features both of smooth muscle cells and fibroblasts and are known to take part in wound healing and contraction (17). They are expressing cytoskeletal proteins like α-smooth muscle actin, vimentin and, to a

lesser extent, desmin. The origin of these cells is a matter of speculation as they are thought to be derived from pericytes, fibroblasts or smooth muscle cells (17). The cytokine transforming growth factor-β (TGF-β), known to be a strong renal fibrogenic growth factor, has been shown to be the most potent activator of myofibroblasts in vivo and in vitro (18). Vimentin is a filament protein that can be found in smooth muscle cells and in glomeruli in the normal kidney, but not in normal adult tubular cells. It is a marker of dedifferentiation and appears in the repair process in damaged renal tubular cells. It disappears when healing is complete (19).

The present study was designed to describe and quantitate the glomerular and interstitial inflammatory cells in renal biopsies from patients with Wegener`s granulomatosis and renal involvement, and also to quantitate markers of early fibrogenesis. Our goal was to study whether any of these cell types and markers correlated with the severity of the renal disease at the time of biopsy and if their presence had any bearing on renal prognosis.

Materials and methods

Patients

One hundred and eight patients with Wegener`s granulomatosis (WG) and active renal disease who were treated in eight hospitals in Norway between 1988 and 1998 were included in this study. Each of the eight hospitals treated all patients with Wegener`s granulomatosis in its catchment area. All hospitals had an electronically based register for patient diagnosis, and records from these registers were used to find all patients who had been given the diagnosis of Wegener`s granulomatosis. The information was then validated by one of the authors (K.A), to confirm the diagnosis.

The review covered all medical information available in the hospital records for each patient, and included an interview with the physicians who treated the patients.

Clinical diagnosis

The diagnosis of WG was based on the clinical criteria developed by the American College of Rheumatology (20). As an additional requirement, patients without proven granulomatous inflammation in a biopsy from any tissue, had to be seropositive for ANCA. A clinical diagnosis of active renal disease was defined as signs of active urine sediment, i.e. more than 5 erythrocytes per high-power field (magnification, x400), or erythrocyte or granular casts.

Renal biopsies

Ninety-four of the 108 patients (60 males and 34 females) had a percutaneous renal biopsy taken on admission to the hospital. The biopsies were sent to the Norwegian Kidney Register where they were examined. The standard light microscopy of the renal biopsies was carried out on sections from paraffin embedded material, and the results from this study has been reported elsewhere (10).

For 61 of the patients additional paraffin embedded material was available for immunostaining in the present study. For comparison, normal appearing tissue from ten kidneys resected for localised neoplasms were included.

Immunohistochemistry

The DAKO Tech Mate 500 slide processing equipment was used. 5 µ sections from formalin fixed, paraffin embedded material were deparaffinised with graded ethanols and blocked with 1% hydrogen peroxide in methanol. Protease and/or heat pretreatment in 10 mmol/L citrate buffer were performed when recommended. After

incubation with the primary antibodies, antigen localisation was revealed by the standard avidin-biotin-peroxidase method. Harris hematoxylin was used for counterstaining.

The *monoclonal antibody CD68*, clone PG-M1 (DAKO code no. M 0876) detects an antigen mainly located on lysosomal membranes after antigen retrieval by heating in citrate buffer. In formalin fixed, paraffin embedded material, the antibody labels macrophages. It does not react with granulocytes. *Polyclonal rabbit anti-human CD3 antibody* from DAKO (code no. A 0452) is considered a highly specific marker for T – lymphocytes, reacting with the intracytoplasmatic portion of the CD3 molecule, which is a part of the T-cell receptor complex. No other cells, except for Purkinje cells, are known to express the CD3 antigen. The *mouse monoclonal antibody CD8*, clone 1 A5, (Novocastra NCL-CD8-295) detects the CD8 co-receptor for MHC class I molecules found on cytotoxic/suppressor T-lymphocytes and also on some natural killer (NK) cells. The DAKO *mouse monoclonal antibody CD20*, clone L 26 (code no. M 0755) is directed against a membrane antigen, probably a calcium channel, present on B-lymphocytes. It is specific for B-lymphocytes and does not react with other hematopoietic cells. *Anti-human smooth muscle actin* clone 1A 4 (DAKO code no. M 0857), is a monoclonal mouse antibody reacting with the α-smooth muscle isoform of actin in smooth muscle cells, myoepitelial cells, pericytes and myofibroblasts. The *rabbit anti-human Ki-67 antibody* (DAKO code no. A0047), is an affinity-isolated antibody that has been shown to work on formalin fixed, paraffin embedded sections. It reacts with a nuclear antigen in proliferating cells after antigen retrieval by heating the tissue in citrate buffer. *Mouse anti-swine vimentin monoclonal antibody* (DAKO code no. M0725), marks intermediate filament proteins mainly in cells of mesenchymal origin. It is also expressed in regenerating tubular epithelial cells.

The slides were coded and examined by one observer (KA) in a blinded protocol, where only the laboratory identification number was available for the investigator. For the analysis of glomerular leukocytes the mean number of positive cells counted in all glomeruli in the biopsy were expressed as cells per glomerular cross section (c/gcs). Cells within crescents were included in the intraglomerular counts. Leukocytes in the interstitium were expressed as the average number of positive cells in 8 fields (magnification x400) giving cells/ 0.15 mm^2. The degree of immunohistochemical positive staining for glomerular, periglomerular and interstitial α-smooth muscle actin (α-SMA) and for tubular cell vimentin was expressed semiquantitatively on a four point scale where 0 was no expression 1, mild expression 2, moderate expression and 3 was strong expression. All glomeruli in the biopsy were evaluated for α-SMA immunostaining, and the average score was recorded for each biopsy. To identify actively proliferating renal tubular cells the number of Ki67 positive tubular cells counted in 20 tubular sections was recorded.

As the amount of material was limited, immunostaining could not be carried out on all 61 samples for all of the markers.

Laboratory investigation

The patients were included in the study at the time of the kidney biopsy. Serum creatinine was used as an estimate of glomerular filtration rate and recorded at inclusion and after one year. For patients on dialysis therapy, serum creatinine was arbitrarily set to 600 μmol/l.

Treatment

At the time of biopsy a total of 18 patients (29.5%) received immunomodulating therapy. Fourteen patients (23 %) received corticosteroids alone,

four patients (6.6%) received corticosteroids in combination with oral or intravenous cyclophosphamide. Duration of therapy at the time of biopsy was less than three days for 13 (72.2%) of the 18 patients receiving treatment. Forty-three patients (70.5%) got no specific treatment for WG at the time of biopsy. Following biopsy, therapy consisted of intravenous or oral cyclophosphamide in combination with corticosteroids for 56 (91.8%) of the patients and corticosteroids and azathioprine for four (6.5%) of the patients. One patient died before treatment was instituted. The treatment protocol included the use of cytotoxic agents (azathioprine or cyclophosphamide) for one year following complete remission after which the drugs were slowly tapered. In the event of a relapse induction therapy with cyclophosphamide and corticosteroids was reinstituted.

Statistical analysis

Spearman`s rank correlation coefficient (ρ) was used to test a possible association between the number of glomerular and interstitial $CD3^+$, $CD8^+$ and $CD68^+$ cells and serum creatinine at the time of biopsy and after one year. Comparing two groups of normally distributed variables was done by student T-test and by a Mann-Whitney U-test in the case of skewed data. A non-parametric one way analysis of variance (Kruskal-Wallis test) was employed to evaluate the association between serum creatinine and the four levels of staining for α-SMA and vimentin. For the correlation analyses with several calculations using the same outcome variable (serum creatinine concentration), the level of significance was set to $P< 0.0035$ according to the Bonferroni method (21). For the rest of the analysis the level of significance was $P< 0.05$. All tests were two-tailed.

Results

The clinical and morphological characteristics of the 61 patients in the study are shown in table I. There were varying numbers of crescentic glomeruli in the biopsies, and in 33 (54 %) of the biopsies 50 % or more of the glomeruli contained crescents fulfilling the WHO criterion for crescentic glomerulonephritis (22).

Inflammatory cells

The vast majority of the inflammatory cells were $CD68^+$ cells (macrophages), but there was also an accumulation of T lymphocytes in glomeruli with crescents (Table II). $CD8^+$ cells constituted 67.7 % of the total T-cell population ($CD3^+$ cells). There was an equal number of $CD3^+$ and $CD68^+$ cells distributed in the interstitial areas. $CD68^+$, $CD3^+$ and $CD8^+$ cells were abundant in the periglomerular areas, especially around glomeruli with crescents (Figure 1 and 2). No intraglomerular $CD20^+$ cells (B-lymphocytes) were found, but were seen in the interstitium, mainly in areas of fibrosis.

The number of macrophages both in the glomeruli and in the interstitium correlated significantly with serum creatinine at the time of biopsy but not with serum creatinine after one year (Table III). The number of interstitial $CD3^+$ cells and interstitial $CD8^+$ cells correlated also to serum creatinine at biopsy but not at one year. There was a tendency towards a correlation between intraglomerular $CD8^+$ cells and serum creatinine after one year, but the correlation did not reach statistical significance ($P = 0.017$). The number of interstitial $CD3^+$, CD 8^+ and $CD68^+$ cells correlated significantly with the fraction of glomeruli with rupture of Bowman`s capsule ($\rho = 0.42$, $P = 0.004$ and $\rho = 0.34$, $P = 0{,}028$ and $\rho = 0.38$, $P = 0.005$), but the intraglomerular number of the same cells did not correlate to the fraction of glomeruli with rupture of Bowman`s capsule (results not shown).

α-SMA and vimentin

Staining for α-SMA was strongly positive in artery walls both in patients and controls. The staining for α-SMA in the glomerulus in patients with WG was weak and not significantly different from tissue obtained from control patients. Some, but not all crescents stained positive, especially where breaks in Bowman`s capsule was noted . However, when all glomeruli including the crescent were evaluated, the α-SMA expression was still low, 1.25 ± 0.82 in patients compared to 0,90 ± 0.57 in controls P =0.197. Periglomerular staining for α-SMA was prominent in all WG patients, especially around glomeruli containing crescents, average score being 1.75 ± 0.78 vs. 0.70 ± 0.67 in controls (P = 0.001) (Figure 3). No patients were characterised as having no (0) α-SMA staining in the interstitium, and average score was 2.03 ± 0.75 vs 1.3 ± 0.82 in controls (P = 0.022). In the Kruskall-Wallis analysis, serum creatinine at the time of biopsy differed significantly among the three levels of interstitial α-SMA staining (Figure 4), and after one year patients belonging to the group with the strongest staining of α-SMA had significantly higher serum creatinine than patients in the two other groups.

Vimentin was expressed in the glomerulus both in normal controls and in patients. Tubular cells also showed varying degrees of positivity for vimentin in the patients (Figure 5). No patients were characterised as having no (0) tubular staining for vimentin. Serum creatinine at biopsy was different in the three levels of tubular vimentin staining at the time of biopsy, but no difference was observed after one year (Figure 6). Tubular vimentin staining was significantly higher in patients with than in patients without signs of acute tubular damage (2.1 ± 0.82 vs.1.7 ± 0.70, P = 0.043).

Ki-67

Ki-67 was frequently expressed within crescents and in periglomerular areas in crescentic glomeruli. It was not seen within the glomerular tuft in normal glomeruli or in glomeruli with crescents. Ki-67 was expressed in some tubular cells, but the counts did not correlate to serum creatinine at biopsy or after one year (results not shown).

Discussion

The dominating inflammatory cells in the renal biopsies were $CD68^+$cells (macrophages) and $CD3^+$ lymphocytes. $CD8^+$ lymphocytes constituted more than 2/3 of the $CD3^+$ cells, indicating that cytotoxic T-cells are the most numerous sub-population of lymphocytes in the glomeruli of patients with WG. Glomerular and interstitial infiltration of macrophages were highly correlated to loss of glomerular function at the time of biopsy, but not after one year. Interstitial $CD3^+$ cells and interstitial $CD8^+$ cells also correlated with initial glomerular function. No B-lymphocytes were identified in the glomeruli. Impairment of renal function at the time of biopsy was greatest in patients with strong expression of interstitial α-SMA and with strong expression of vimentin in renal tubular cells. The detection of strong interstitial α-SMA expression indicated high serum creatinine one year after renal biopsy.

The important role for macrophages in crescent formation has long been known (23). Macrophage presence and proliferation are prominent in rat crescentic glomerulonephritis, but not in normal rat kidney or in mild glomerular injury induced by hypercholesterolemia (24). In man, glomerular macrophage infiltration in ANCA associated glomerulonephritis has been shown to be extensive, and found exclusively in areas of necrosis, where it is co-localised with adhesion molecules (ICAM-1 and

VCAM-1) (25). Macrophages are established cytotoxic effector cells in cellular immune responses and can upon activation release oxygen radicals and lysosomal enzymes that damage adjacent cells (26). They can also synthesise and release a variety of cytokines and growth factors that, in turn, attract more infiltrating immune cells and activate resident cells, thereby amplifying the tissue injury (24, 27). Despite the highly significant correlations between the number of interstitial and glomerular macrophages and serum creatinine at the time of biopsy found in the present study, the macrophage induced acute renal impairment was largely reversible by treatment, as no correlation to longterm renal outcome could be shown.

We did not find evidence for an antibody mediated glomerulonephritis. We have shown in an earlier study that there were few or no signs of antibody deposition in the biopsies (10), and $CD20^+$cells (B-lymphocytes) were not present in the glomeruli, although they could be identified in the interstitium, especially in areas of fibrosis. T-cells however, were numerous both in the glomerulus and in the interstitium, and a dominating macrophage and $CD3^+$ accumulation suggest a type IV immune response in our patients with WG.

There is substantial experimental evidence that T cells can induce acute glomerular injury even in the absence of glomerular antibody deposition (28). The importance of T-cells in crescent formation was demonstrated in a model of crescentic glomerulonephritis in WKY rats where an antibody blocking the CD28/B7 costimulatory-signal for T-cell activation led to a marked reduction in crescent formation, even when it was administered after the disease was established (29). Studies in human glomerulonephritis have also indicated an association between T-cells accumulating in glomeruli and crescentic glomerulonephritis (30, 31), but the role of T cells in the pathogenesis of ANCA associated systemic vasculitis is uncertain (32). Autoreactive PR3 specific T-cells are present in the blood of patients

with WG (33), and peripheral blood T-lymphocytes from patients with ANCA associated vasculitis have an increased expression of T cell activation markers, regardless of disease phase or immunosuppressive therapy (34).

$CD4^+$ lymphocytes are usually the most numerous T-cells found in type IV cellular infiltrates (15), and in a recent study $CD4^+$ cells were found to have a pivotal role in the crescentic lesion in experimental anti-GBM glomerulonephritis in mice. In the absence of $CD4^+$ cells no crescents developed, but CD8-deficient animals developed severe crescentic glomerulonephritis (35). In contrast to these findings, two studies of crescentic glomerulonephritis in rats indicated a crucial role for $CD8^+$ cells in crescent formation (36, 37). Depletion of circulating $CD8^+$ cells was followed by decreased glomerular expression of intercellular adhesion molecules (ICAM-1), cytokines, chemokines and of glomerular monocyte/macrophage accumulation.

In the present study the majority of intraglomerular T cells were $CD8^+$ lymphocytes. A reduced blood CD4/CD8 ratio in WG patients has been shown by other groups (38, 39). In a recent series, patients with generalised WG had evidence of an increased fraction of $CD8^+$ lymphocytes lacking the co-stimulatory molecule CD 28 (40). There was good correlation between a marker of disease activity and the fraction of $CD8^+/CD28^-$ cells but not between disease activity and $CD4^+/CD28^-$ cells, indicating an important role for $CD8^+/CD28^-$ lymphocytes in the pathogenesis of WG. Activation of lymphocytes as shown in systemic vasculitis (34) will lead to enhanced production of cytokines and chemokines, which in turn increase the expression of adhesion molecules on glomerular endothelial cells and circulating leukocytes (37, 41). This will result in accumulation of macrophages and leukocytes in the glomerular tuft, eventually inducing necrotizing capillary damage (42), an event supposed to precede crescent formation (43).

We found a significant correlation between the number of interstitial glomerular inflammatory cells ($CD3^+$, $CD8^+$ and $CD68^+$) and breaks in Bowman`s capsule. Rupture of Bowman`s capsule is a common feature of crescentic glomerulonephritis, and morphological studies have indicated that periglomerular fibroblasts, macrophages and T-cells can migrate into the glomerulus through the breaches, probably attracted by chemotactic factors originating from the glomerular inflammatory cells (44-46). A direct involvement in the rupture of Bowman`s capsule by periglomerular mononuclear cells, even in the absence of glomerular crescents, has been suggested in a study of experimental anti GBM disease in rats (47). We observed a marked periglomerular infiltration of T-lymphocytes and macrophages around glomeruli containing crescents, and although our findings merely represents an association, it could suggest a role for interstitial leukocytes in Bowman`s capsule rupture in human pauci-immune crescentic glomerulonephritis as well.

In the present study, the highest serum creatinine concentration, both at the time of biopsy and at one year, was found in patients with the strongest expression of interstitial α-SMA. α-SMA is expressed by myofibroblasts, which are cells sharing features both of smooth muscle cells and fibroblasts. They express a wide range of cytoskeletal proteins (17). Transforming growth factor-beta (TGF-β) has been found to be involved in the activation and differentiation of myofibroblasts (18) and collagen synthesis in renal cortex in rats was highly correlated to increased activity of TGF-β (48). Increased expression of TGF-β in α-SMA positive cells in experimental renal scarring (16) indicates that this cytokine is of importance in driving the fibrotic process in kidney disease, and our findings support a possible role for myofibroblasts in this process in this cohort of patients.

Mesangial cells have also been shown to express α-SMA following experimental glomerulonephritis (49). Similar phenotypic changes are reported in

human mesangioproliferative glomerulonephritis (50). Mesangial proliferation is not a prominent feature of ANCA-associated crescentic glomerulonephritis, and this may be the reason for the lack of α-SMA expression in the glomerulus in our study. There are, however, reports of increased glomerular expression of α-SMA in crescentic glomerulonephritis, mainly in the crescents (50, 51). The glomerular α-SMA expression was not significantly different from normal controls in the present study, although staining for α-SMA was increased in some of the crescents. Expression of α-SMA in crescents may be a sign of early fibrogenesis, which eventually will result in sclerotic glomeruli. In all patients there was a marked periglomerular staining, suggesting myofibroblast accumulation in this area.

Tubular vimentin expression was higher in patients with than in patients without signs of acute tubular damage. Additionally, tubular vimentin expression correlated significantly with serum creatinine at the time of biopsy but not after one year, suggesting that acute tubular damage may be responsible for part of the reduction in GFR at the time of renal biopsy. These observations are in keeping with a report of increased vimentin expression in regenerating rat proximal tubular cells in response to acute injury (52). Transient vimentin expression is a marker of regenerating and proliferating tubular cells (19), whereas persistent expression may indicate chronic renal damage. Vimentin analysis in repeat biopsies could be a method for the testing of this hypothesis.

In conclusion, we have found macrophages to be the most numerous infiltrating cells and $CD8^+$ lymphocytes to be the largest subset of lymphocytes in the glomeruli of patients with WG and renal involvement. Accordingly, treatment of WG should be aimed at inhibiting the accumulation and proliferation of T-cells and macrophages within the glomerulus. Christensson and co-workers (34) suggested that the current therapy may be insufficient in this respect, as they found activation of T-

lymphocytes, including $CD8^+$ cells, to persist even when the patients were receiving treatment with corticosteroids and cyclophosphamide. Cytokines and cell surface proteins specifically modulating T cell activation and macrophage infiltration, may be attractive targets for immune modulating therapy (53-55). Blocking the T-cell activation by inhibiting cell surface proteins (B7, CD2) or inhibiting stimulatory cytokines (IL-1, IL-2, IL-12, IFN-γ), are strategies worth exploring in the future.

To inhibit or reduce the sclerotic process in the kidneys could also be an important objective for further research. Suppression of extracellular matrix production by connective tissue cells like myofibroblasts has been demonstrated with the use of antiserum against TGF-β in one model of experimental glomerulonephritis (56). Local delivery of decorin, a natural inhibitor of TGF-β, into the kidneys also reduced matrix deposition significantly in the same model (57). Oral antagonists to TGF-β has become available and represent promising agents for reducing the sclerotic process. Mycophenolate mofetil treatment has been shown to markedly reduce myofibroblast infiltration, resulting in attenuated collagen III deposition in a rat remnant kidney model (58). The further exploitation of these and other strategies in order to find treatments for systemic vasculitis, may bring about a better prognosis for our patients.

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Table I. Clinical and biopsy data at initial evaluation in 61 patients with Wegener`s granulomatosis and renal involvement

Age years median (range)	57 (15 –80)
S creatinine (μmol/l) median (range)	243 (53-1356)
U protein g/24 h median (range)	1.5 (0 – 4.7)
C-ANCA *n* (%)[a]	43 (79.6)
P-ANCA *n* (%)[b]	5 (9.3)
Dialysis at inclusion *n* (%)	14 (23)
Median number of glomeruli in biopsies (range)	11 (4 –50)
Fraction of normal glomeruli % (range)	31 (0 –100)
Number of biopsies with crescents n (%)	54 (88.5)
Number of biopsies with glomerular necrosis *n* (%)	51 (83,6)

[a]C-ANCA, cytoplasmic antineutrophil cytoplasmic autantibodies; [b]P-ANCA, perinuclear antineutrophil cytoplasmic autoantibodies; The percentage of crescents is expressed as a fraction of the number of nonsclerotic glomeruli in the biopsies; The percentage of normal glomeruli is expressed as a fraction of the total number of glomeruli in the biopsies. 54 patients initially tested for ANCA

Table II. Mean (±SD) number of glomerular and interstitial leukocytes in the renal biopsies

Cell type	Glomerulus[a]	Interstitium[b]
$CD68^+$	29.9 (±15)	42.6 (±28)
$CD3^+$	2.57(±1.8)	48.0 (±32)
$CD8^+$.	1.74 (±1.2)	22.1 (±16)
$CD20^+$	–	14.5 (±13)

[a] cells per glomerular cross section (c/gcs) [b] cells per 400x field (0.15 mm^2)

Table III. Correlation between number of glomerular and interstitial leukocytes and serum creatinine at biopsy and after one year.

Marker	S-creatinine at biopsy		S-creatinine after one year	
CD68 glomerular	$\rho^a = 0.489$	P = 0.001*	$\rho = 0.302$	P = 0.073
CD68 interstitial	$\rho = 0.580$	P = 0.000*	$\rho = 0.251$	P = 0.062
CD3 glomerular	$\rho = 0.362$	P = 0.042	$\rho = 0.091$	P = 0.965
CD3 interstitial	$\rho = 0.473$	P = 0.001*	$\rho = 0.262$	P = 0.078
CD8 glomerular	$\rho = 0.392$	P = 0.026	$\rho = 0.424$	P = 0.017
CD8 interstitial	$\rho = 0.529$	P = 0.000*	$\rho = 0.316$	P = 0.039
CD20 interstitial	$\rho = 0.260$	P = 0.065	$\rho = 0.168$	P = 0.254

[a]Spearman`s rank correlation coefficient * Significance P< 0.0036

Figure 1. Distribution of CD68 positive cells. Monocytes/macrophages localises to a cellular crescent and are also present in increased amounts in the interstitium, especially close to the glomerulus. Original magnification x 370.

Figure 2. Accumulations of CD8 positive cells in the interstitium mainly adjacent to Bowman's capsule. Some positive cells are seen in the urinary space of a glomerulus, colocalising with a cellular crescent. Original magnification x 353.

Figure 3. α-smooth muscle actin staining predominantly located periglomerular. Spindled cells adjacent to Bowman's capsule and also a few cells in the crescent, express α-smooth muscle actin. Original magnification x 560.

Figure 4. Boxplots showing serum creatinine as a function of three levels of interstitial α-smooth muscle actin expression in renal biopsies, at the time of biopsy (Panel A) P = 0.0001 and one year after the biopsy (Panel B). P = 0.032 (Kruskal-Wallis test).

Groups; 1, mild expression 2, moderate expression and 3, strong expression. The box exstends from the 25th to the 50th percentile. The line is the median value. Whiskers extend to lowest and highest observed values within 1.5 box lengths. Plus signs, observations between 1.5 and 3 box lengths. Asterisks, observations more than 3 box lengths from the upper edge of the box

Figure 5. Vimentin localising to epithelial cells of some proximal tubules. The glomerulus shows strong expression in most of the cells in the cellular crescent and there is some positivity in the glomerular tuft. Original magnification x 350.

Figure 6. Boxplots showing serum creatinine as a function of three levels of tubular vimentin expression in renal biopsies, at the time of biopsy (Panel A) P = 0.0001 and one year after the biopsy (Panel B). P = 0.47 (Kruskal-Wallis test).

Groups; 1, mild expression 2, moderate expression and 3, strong expression. The box exstends from the 25th to the 50th percentile. The line is the median value. Whiskers extends to lowest and highest observed values within 1.5 box lengths. Plus signs, observations between 1.5 and 3 box lengths. Asterisks, observations more than 3 box lengths from the upper edge of the box.

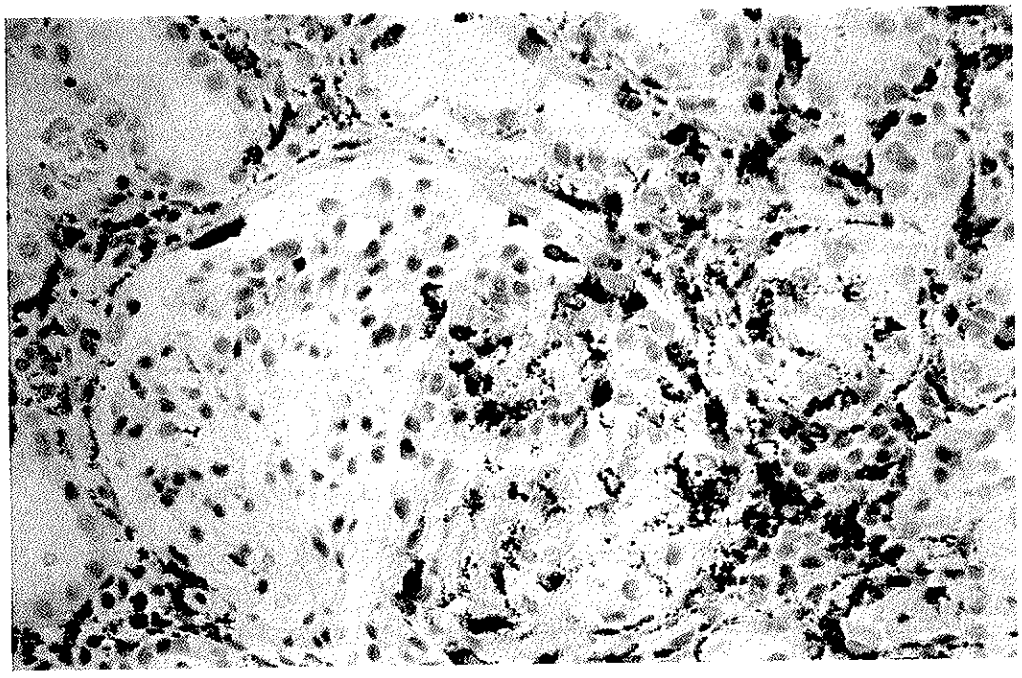


Figure 1

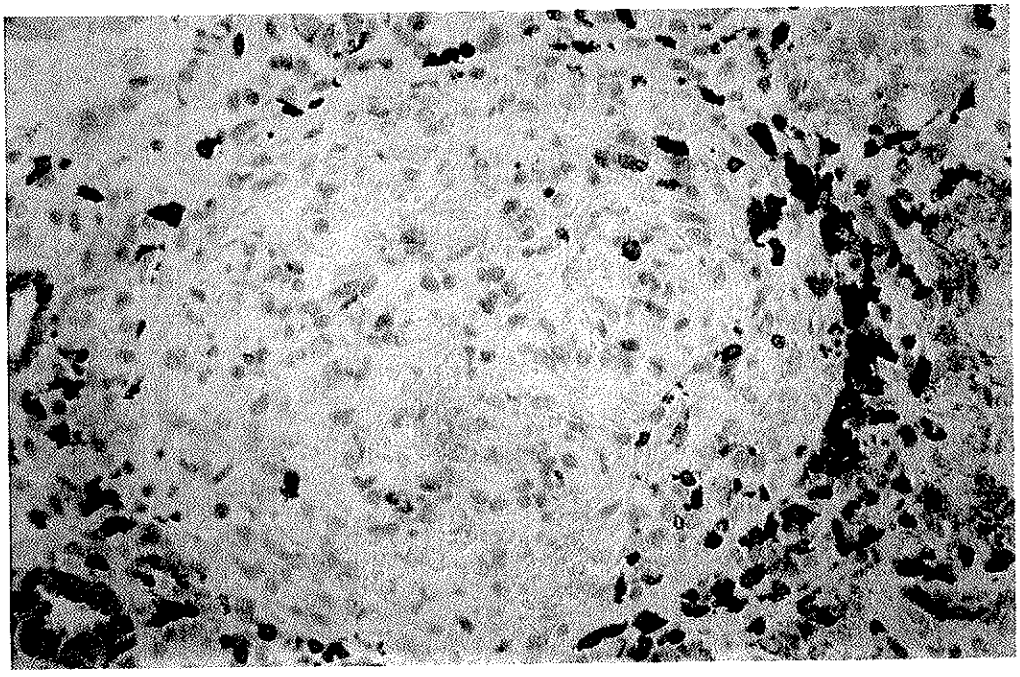


Figure 2

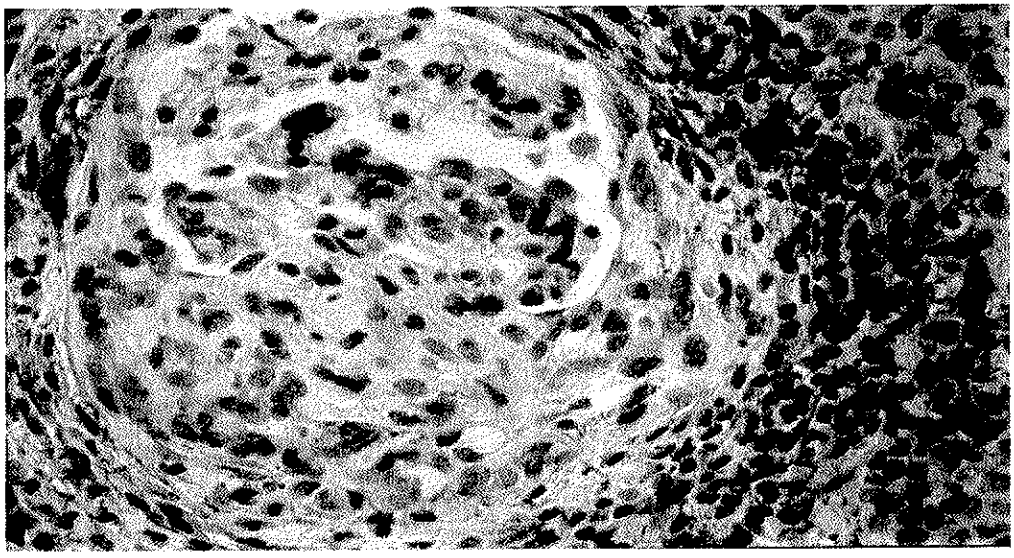
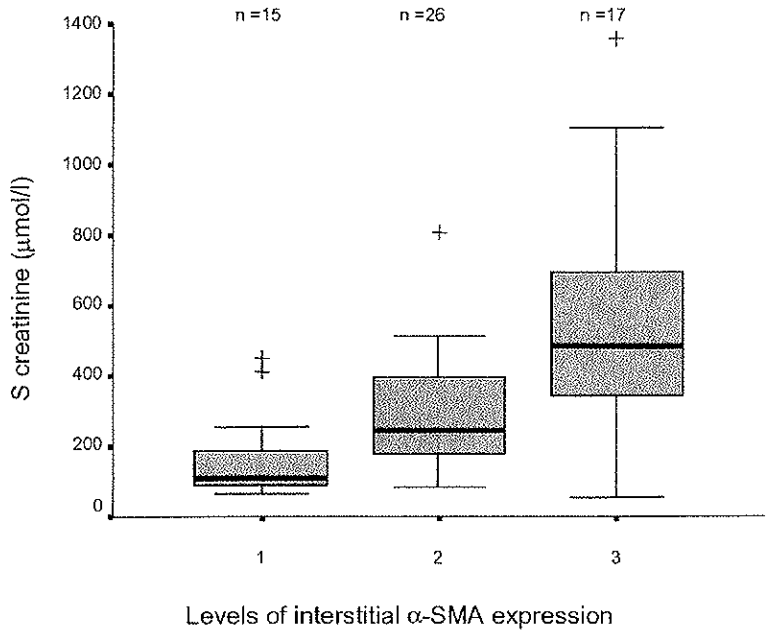


Figure 3

A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n =26
n =17
1400
1200
1000
800
600
400
200
0
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1
2
3
Levels of interstitial α-SMA expression

B

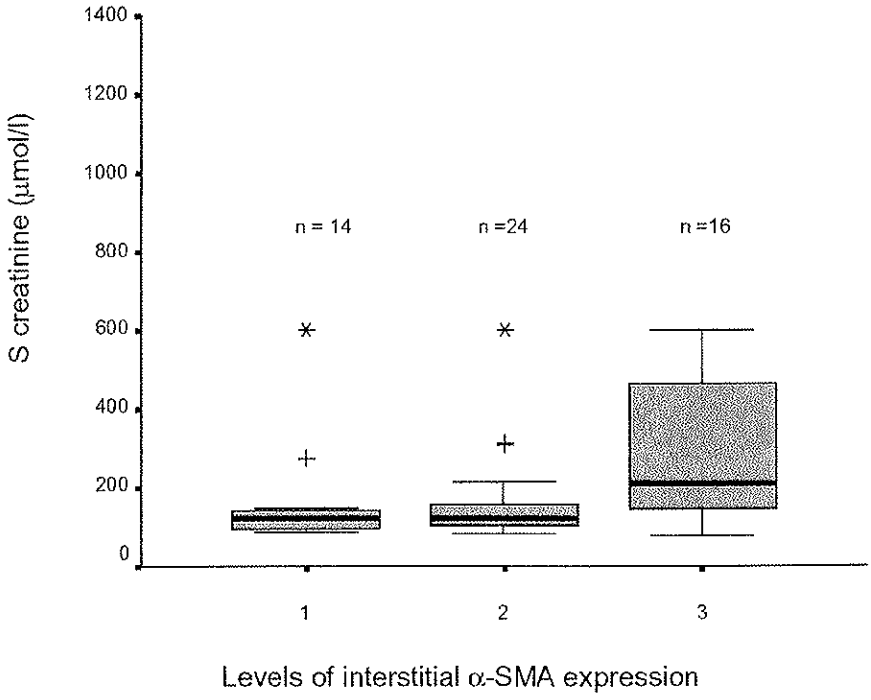

1400
1200
1000
800
600
400
200
0
S creatinine (µmol/l)
n = 14
n =24
n =16
1
2
3
Levels of interstitial α-SMA expression

Figure 4

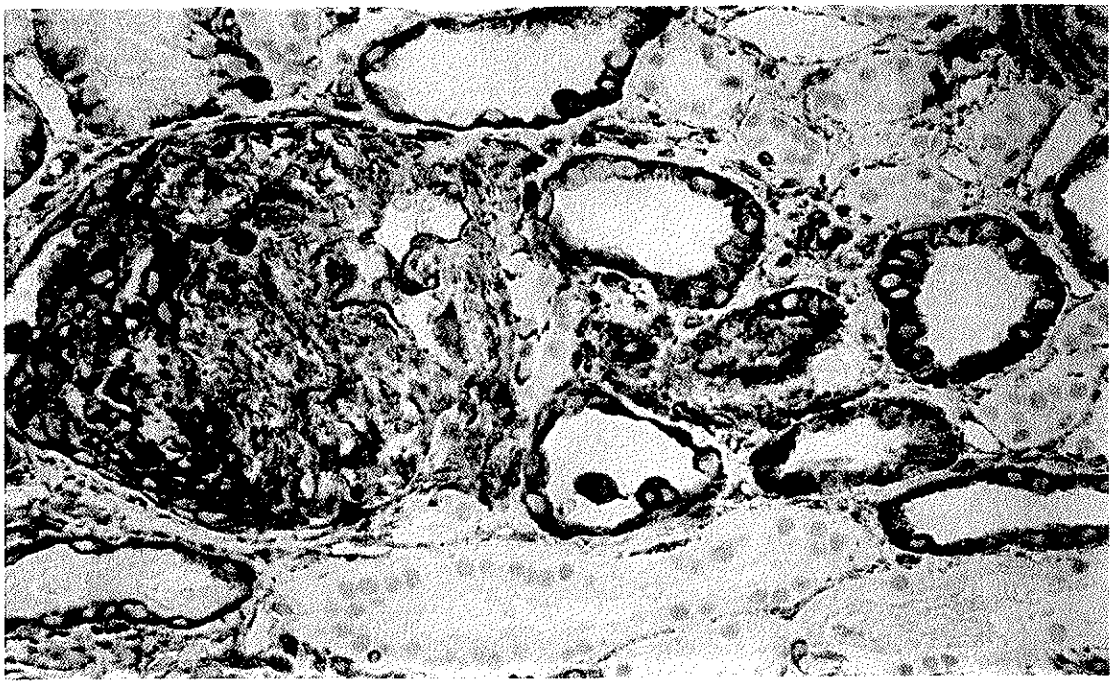
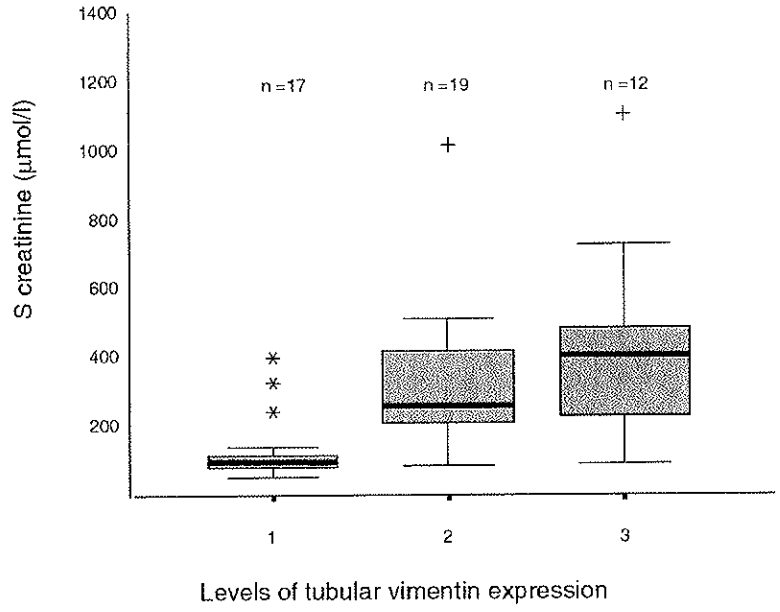


Figure 5

A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1200
1000
800
600
400
200
S creatinine (µmol/l)
n =17
n =19
n =12
1
2
3
Levels of tubular vimentin expression

B

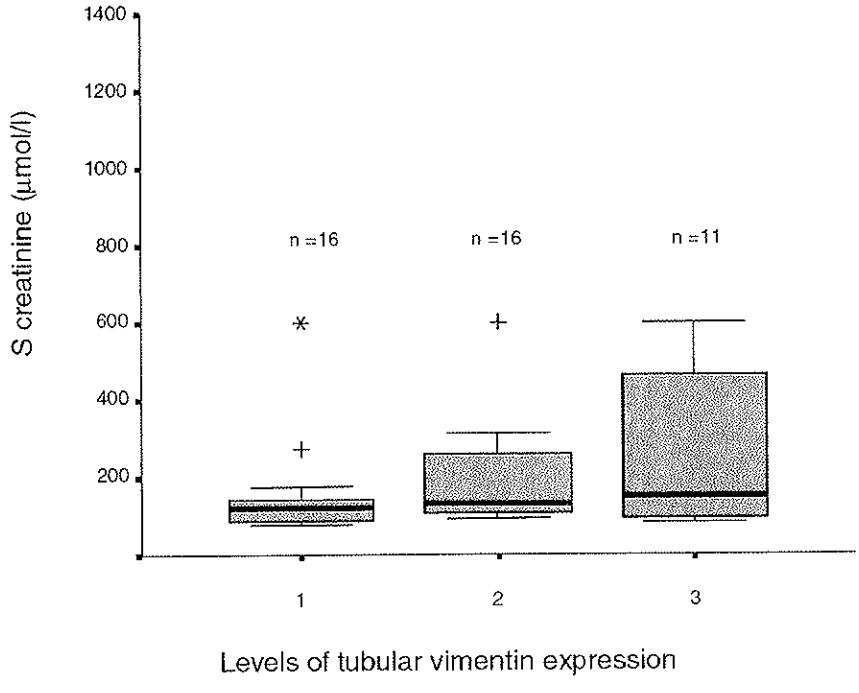
1400
1200
1000
800
600
400
200
S creatinine (µmol/l)
n =16
n =16
n =11
1
2
3
Levels of tubular vimentin expression

Figure 6

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